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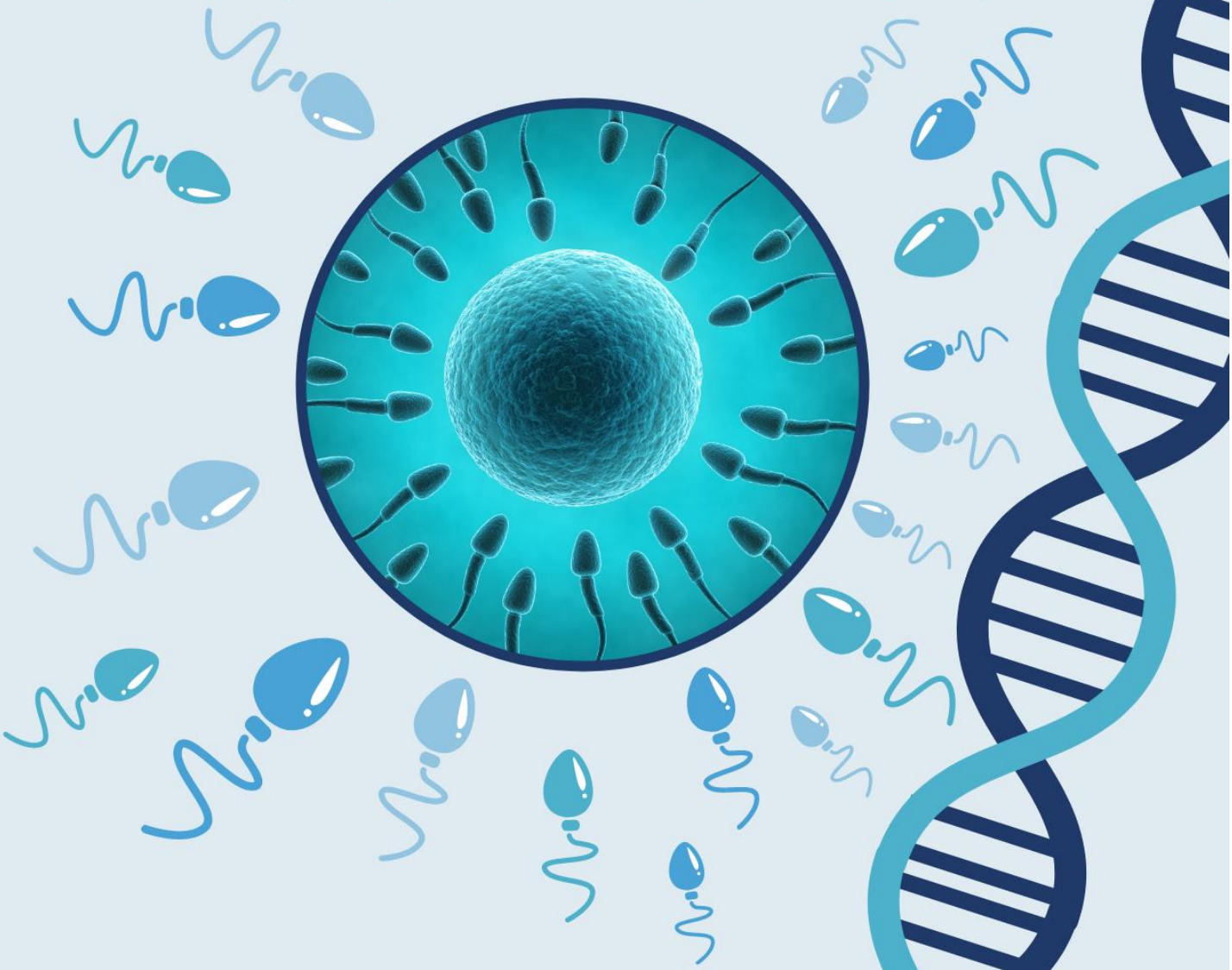
Εργαστήριο Γενετικής, Συγκριτικής και Εξελικτικής Βιολογίας

# Ανάλυση και συσχέτιση πολυμορφισμών των γονιδίων της γλυκόλυσης και της οξειδωτικής φωσφορυλίωσης (OXPHOS) με την ανδρική υπογονιμότητα στον Ελληνικό πληθυσμό



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**«Ανάλυση και συσχέτιση πολυμορφισμών των γονιδίων της γλυκόλυσης και της οξειδωτικής φωσφορυλίωσης (OXPHOS) με την ανδρική υπογονιμότητα στον Ελληνικό πληθυσμό» – "Polymorphism analysis and association of glycolysis and oxidative phosphorylation (OXPHOS) genes with male infertility in the Greek population"**

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# Ευχαριστίες

Τελειώνοντας τη συγγραφή της παρούσας εργασίας θα ήθελα να ευχαριστήσω έναν έναν ξεχωριστά όλους όσους συνέβαλλαν στην πραγματοποίησή της και με βοήθησαν με κάθε τρόπο.

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# Abstract

Infertility is a major public health problem that affects many couples worldwide and can lead to a variety of economic, psychological, and social problems. However, 50% of all infertility cases are due to male factor. Male infertility is a complex disorder affected by environmental and genetic factors. For events required during spermatogenesis and fertilization a great amount of energy is used. The two main pathways that provide cells with ATP are the Glycolysis pathway and Oxidative Phosphorylation (OXPHOS). In this study, blood and semen samples from normozoospermic and non-normozoospermic individuals were used to perform a Genome-Wide Association Study (GWAS) and Whole-Genome Sequencing (WGS) analysis in order to identify then, variants on glycolysis and OXPHOS genes associated with male infertility and specific subtypes of male infertility (azoospermia, oligozoospermia, teratozoospermia). More specifically, sperm from normozoospermic and non-normozoospermic men of the Greek population was used for SNP genotyping, and after that, four association analyses were performed by comparing different groups of individuals (Normozoospermic vs Non-Normozoospermic, Normozoospermic vs Asthenozoospermic, Normozoospermic vs Oligozoospermic, and Normozoospermic vs Teratozoospermic). For every comparison, statistically significant SNPs found on Glycolysis and OXPHOS genes were selected. Blood samples were also used for WGS and bioinformatics analysis followed to detect unique variants for every group and every comparison performed (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic). As a result, several variants associated with male infertility and its specific subtypes were detected. Of particular interest is the fact that an association was observed by WGS analysis between asthenozoospermia and variants on Glycolysis genes. Both nuclear and mitochondrial-encoded OXPHOS genes also seem to be involved in male infertility pathogenesis. Therefore, exploring the association between sperm metabolism and bioenergetics and male infertility may contribute to the understanding of its molecular mechanisms and the mechanisms underlying the maintenance of fertility. The SNPs identified may also act as a potential biomarker for the clinical diagnosis of specific subtypes of male infertility.

*Keywords:* male infertility, glycolysis, oxidative phosphorylation, energy production, ATP

# Contents

# Introduction

# CHAPTER I: MALE REPRODUCTIVE SYSTEM AND SPERMATOGENESIS PROCESS

Sexual reproduction is the process that involves offspring production by the fusion of male and female gametes. Thus, for this process, both the male and female reproductive systems are required (De Visser & Elena, 2007).

The human reproductive systems include primary and secondary organs. The primary reproductive organs are the gonads which are responsible for producing the gametes and sex-specific hormones (Mohanty & Singh, 2017). The secondary organs promote the gamete's growth, maturity, and transport, whereas in females, they also nurture the developing embryo (Rudmann & Foley, 2018). The reproductive organs can be also classified as external or internal according to their position (Gurung & Jialal, 2019).

## *I.I. Anatomy and Physiology of the Male Reproductive System*

The male reproductive system is complex and consists of many external structures. Testes are the primary reproductive organs in males as they produce sperm. Moreover, the internal structures of the male reproductive system are, except for the paired testes, the epididymis, the vas deferens, the ejaculatory duct, the urethra, the seminal vesicles, the bulbourethral gland, and the prostate gland. The external structures are the penis and the scrotum (Mohanty & Singh, 2017). The main function of the male reproductive system is the production and storage of the male gametes, as well as their transportation to the female reproductive system for the achievement of fertilization. The male reproductive system also produces hormones that promote the development of sexual characteristics and play an important role in sperm production. These hormones are called androgens (Gurung & Jialal, 2019).

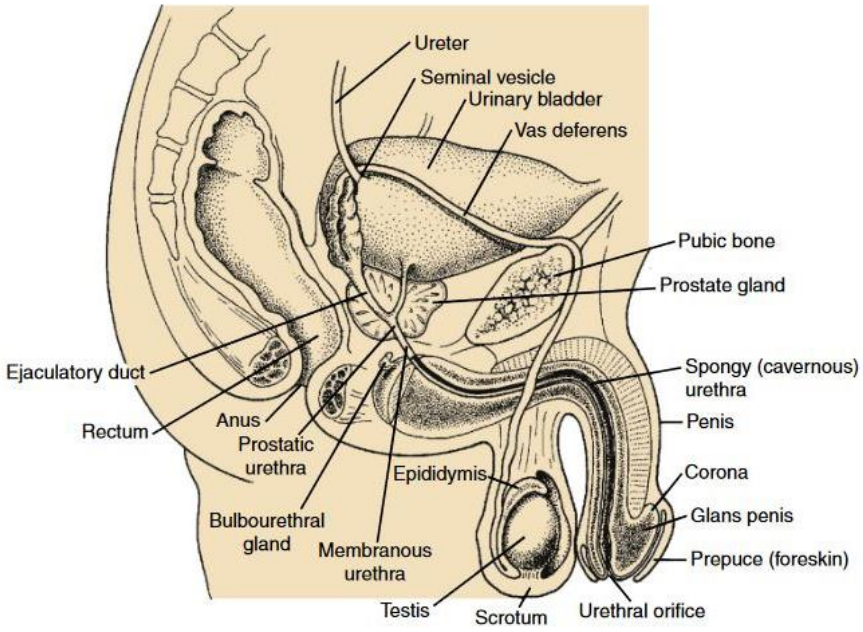


*Figure 1: Overview of the male reproductive system (Jones & Lopez, 2014).*

The scrotum is a thin external sac behind the penis that contains the testes. The scrotal wall consists of a thin layer of skin and smooth muscles, the *tunica dartos*. Under them, there is another layer of muscle, the *cremaster* (Jones & Lopez, 2014; Mohanty & Singh, 2017). The main function of the scrotum is to maintain the ideal temperature for the spermatogenesis process (3.1°C lower than the body temperature) by contraction of the muscles and the movement of the testes further away from the body cavity (Jones & Lopez, 2014).

The two testes, or testicles, are found in the scrotum and they are the male gonads. Their role involves the production of the male sex hormone, testosterone, and sperm production (Jones & Lopez, 2014; Mohanty & Singh, 2017). They are oval-shaped structures and have a length of approximately 4.0 cm. Each testis can be internally divided into approximately 250 compartments or *testicular lobules*. Each lobule contains one or more *seminiferous tubules*, where sperm is formed. Seminiferous tubules are long structures that extended can reach a length of 30-90 cm (Jones & Lopez, 2014). Moreover, within each seminiferous tubule, the seminiferous epithelium comprises male germ cells and Sertoli cells that both play a very important role in the spermatogenesis and spermiogenesis, as it is going to be further analyzed in the next subchapters (Jones & Lopez, 2014; Mohanty & Singh, 2017). Adjacent to the seminiferous tubules, there are also the Leydig cells that produce testosterone by cholesterol found in their cytoplasm in the presence of the luteinizing hormone (LH). After its secretion, testosterone acts in Sertoli cells and regulates sperm production and maturation. Testosterone is also necessary for the development and maintenance of male secondary sex characteristics (Mohanty & Singh, 2017; O'Donnell et al., 2000).

The epididymis, vas deferens, and ejaculatory ducts form the sperm canal. Together they extend from the testis to the urethra and they play an important role in sperm transport (Mohanty & Singh, 2017). The epididymis is found on the backside of each testicle and it consists of a long, coiled tube. It contributes to sperm transport, protection, and storage. Moreover, spermatozoa are fully differentiated when they leave the testis, but they are incapable of fertilization. They gain their functionality during a maturation process that occurs within the epididymis. The vas deferens or ductus deferens is a long tube that extends from the epididymis to the pelvic cavity, behind the bladder. Each vas deferens enters an ejaculatory duct that empties into the urethra (Jones & Lopez, 2014; Mohanty & Singh, 2017).

The male sex accessory glands are the seminal vesicles, prostate gland, and bulbourethral gland. These structures provide secretions to form the bulk of the seminal fluid or semen (Jones & Lopez, 2014). The seminal vesicles are sac-like pouches that are found at the base of the urinary bladder. They secrete a sugar-rich fluid that provides sperm with fuel to move and represents 60% of the semen volume (Mohanty & Singh, 2017). Moreover, the prostate is a walnut-sized structure found below the urinary bladder. It secretes a fluid that becomes part of the seminal plasma and promotes sperm liquefaction. The contraction of its muscles also helps to propel this seminal fluid into the urethra during ejaculation (Mohanty & Singh, 2017; Wilson,

2014). The bulbourethral glands are also called Cowper's glands and are pea-sized structures found on either side of the urethra, just below the prostate gland. They secrete a clean and thick fluid that acts as a lubricant for the urethra and as a neutralizing agent because urine can create an acidic environment in the urethra (Mohanty & Singh, 2017).

The urethra is a tube responsible for the removal of urine from the body. In males, it also ηας the additional role of ejaculating semen and transporting it in the female's genital tract (Jones & Lopez, 2014). Lastly, the penis is the male external genitalia, and it comprises the *body* or *penile shaft* and a cone-shaped end called the *gland penis*. The body consists also of three circular-shaped chambers of spongy tissue that fill with blood when the man is sexually aroused. One of these chambers is called the *corpus spongiosum* and is traversed by the urethra. When the penis is erect, the flow of urine is blocked from the urethra, allowing only semen to be ejaculated (Jones & Lopez, 2014; Mohanty & Singh, 2017).

### *I.II. Spermatogenesis and Spermiogenesis*

Spermatogenesis is a term that encompasses all complex and highly organized processes to produce mature male gametes (Haiqi Chen et al., 2017; Jones & Lopez, 2014; Neto et al., 2016). It can be divided into four major cellular events: (i) proliferation of spermatogonia, (ii) spermatogonial differentiation into spermatocytes, (iii) meiosis, and (iv) spermiogenesis, a transformation of round spermatids to mature spermatozoa (Haiqi Chen et al., 2017; Neto et al., 2016).

The spermatogenesis process begins with spermatogonia, progenitor germ cells found near the basement membrane of the seminiferous tubules (Haiqi Chen et al., 2017; Jones & Lopez, 2014; Mohanty & Singh, 2017). The spermatogonial stem cell population (SSC) divides to maintain a pool of undifferentiated SSC, ensuring a constant supply of spermatogonia, and providing spermatogonia for sperm production (Haiqi Chen et al., 2017; O'Donnell et al., 2000). Spermatogonia multiply by mitosis, a cell division process that results in the production of two diploid daughter cells derived by a diploid parent cell. Spermatogonia destined to become spermatozoa lose their contact with the basement membrane of the seminiferous tubules and enter meiosis. These are called primary spermatocytes (Haiqi Chen et al., 2017; Jones & Lopez, 2014). Meiosis in human males begins in puberty (Haiqi Chen et al., 2017). After the completion of the first meiotic division, secondary spermatocytes are produced. Secondary spermatocytes are haploid cells that undergo a second meiotic division and give rise to four haploid cells called spermatids (Haiqi Chen et al., 2017; Jones & Lopez, 2014). It should also be noted that during meiosis, the meiotic recombination is a mechanism that involves the formation of DNA double-strand breaks (DSBs) and subsequent repair using the homologous chromosomes to ensure the exchange of genetic material and the genetic variability of the gametes (Hegde & Crowley, 2018).

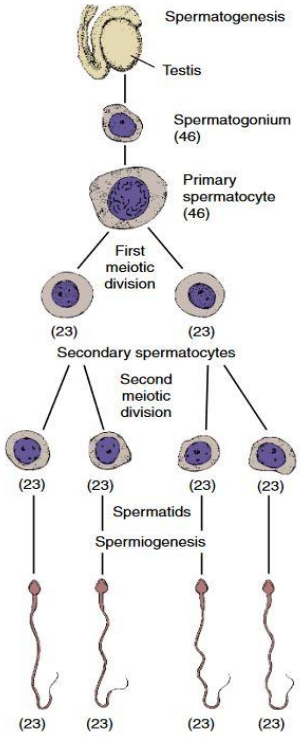


*Figure 2: Spermatogenesis process. In parentheses, the number of chromosomes in each cell type can be found (Jones & Lopez, 2014).*

When spermatids are formed, the spermiogenesis process begins. During spermiogenesis, round spermatids undergo morphological changes to become mature spermatozoa that have a characteristic structure (*Figure 3*) (Haiqi Chen et al., 2017; Jones & Lopez, 2014; Mohanty & Singh, 2017). More specifically, a mature spermatozoon consists of the head, the neck, the midpiece, and the tail (Mohanty & Singh, 2017; Mortimer, 2018). The head has a compact nucleus and is surrounded by a small amount of cytoplasm. It has also a cap-like structure, the acrosome, that secretes enzymes that assist sperm penetration to the ovum (Mohanty & Singh, 2017). The sperm neck has centrioles that form the flagellum and after fertilization form the major microtubule-organizing center of the zygote (Avidor-Reiss, 2018; Mortimer, 2018). The midpiece is also called the powerhouse of sperm as it possesses 12 or 13 mitochondria that produce ATP for sperm motility. The tail is the longest part (45 to 50 μm long) that is made up of flagella (Mortimer, 2018). Flagella's movement is the basis of sperm motility. For many years it was considered that the tail moves symmetrically in a helical shape. However, recently it was discovered that the tail's movement is much more complex combining asymmetrical standing and traveling waves as well as rotating the entire body to achieve a perceived symmetry (Gadêlha et al., 2020).

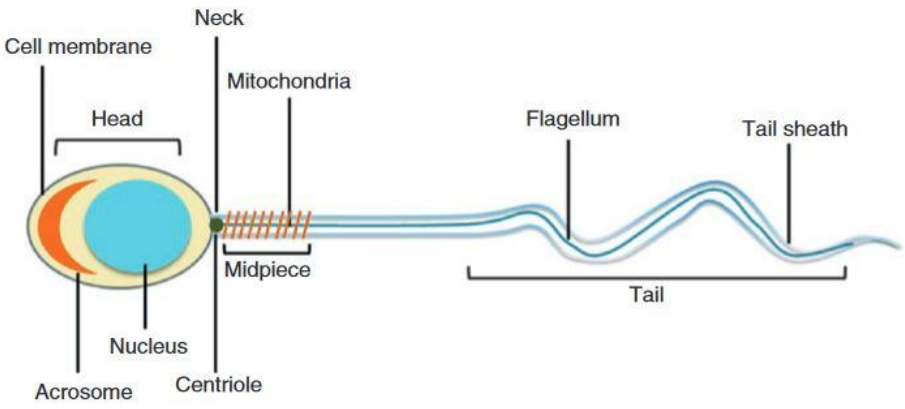


*Figure 3: Structure of human spermatozoon (Mohanty & Singh, 2017).*

Thus, the spermiogenesis process (*Figure 4*) begins with the acrosome biogenesis by the Golgi apparatus (Haiqi Chen et al., 2017; Jones & Lopez, 2014; O'Donnell et al., 2000). After that, the elongation process of spermiogenesis begins with nuclear changes. Spermatid's DNA undergoes packaging and becomes highly condensed. This is achieved by the replacement of histones with protamines. All these changes also cause the inactivation of the gene transcription (O'Donnell et al., 2000). The elongation phase proceeds with the formation of the flagellum. At first, a filamentous structure from one of the pairs of centrioles of the spermatid emerges. Microtubules elongate and form an axoneme. The axoneme contains microtubules that are arranged in a 9 + 2 configuration. This means that a pair of microtubules lies at the center and is then surrounded by 9 outer doublet microtubules (Mortimer, 2018; O'Donnell et al., 2000). Mitochondria cluster around the flagellum and the mid-piece is formed. Finally, the excess cytoplasm, the residual body, is phagocytosed by surrounding Sertoli cells and the remaining cytoplasm and organelles are reorganized (Haiqi Chen et al., 2017; O'Donnell et al., 2000). Spermiation is the last process that involves the release of highly specialized mature spermatozoa from Sertoli cells into the testicular tubule lumen (Jones & Lopez, 2014; Neto et al., 2016).

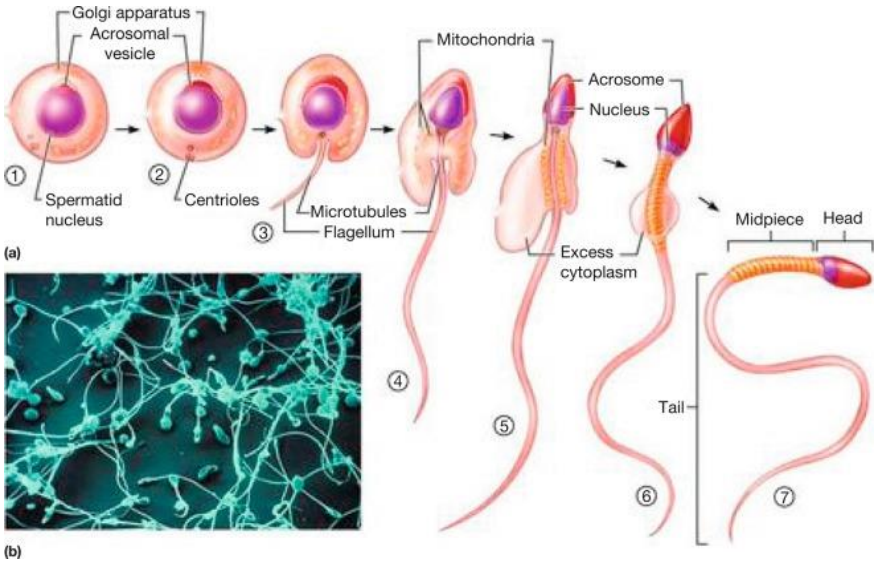


*Figure 4: The spermiogenesis process and changes that occur (Lin & Troyer, 2014).*

It is estimated that the entire spermatogenesis process requires approximately 74 days to be completed (Neto et al., 2016). It is also a highly regulated process affected by hormones, paracrine factors, small non-coding RNAs, genes, etc. Therefore, a lot of factors can affect sperm's quality and quantity (Haiqi Chen et al., 2017; Neto et al., 2016).

### *I.III. Pathway of sperm*

As it has already been described, the testes produce millions of sperm every day. It is estimated that about 4.7 million sperm/g testes are produced every day (Mohanty & Singh, 2017). Sperm is produced during the spermatogenesis and spermiogenesis process in the testes (Haiqi Chen et al., 2017; Jones & Lopez, 2014; Neto et al., 2016) and after that, it follows a specific pathway until it reaches the female genital tract for fertilization to occur (Okabe, 2018). More specifically, spermatids move from the testis to the epididymis and undergo a maturation process (Haiqi Chen et al., 2017; Jones & Lopez, 2014; Neto et al., 2016). The epididymis, vas deferens, and ejaculatory ducts create a transportation system. The vas deferens pushes the sperm up over the bladder and down toward the prostate gland. There, the vas deferens joins the ends of the seminal vesicles to form the ejaculatory ducts. The ejaculatory ducts receive seminal fluid from the vesicles, pass through the prostate, and move semen into the urethra (Jones & Lopez, 2014; Mohanty & Singh, 2017). The male urethra extends from the bladder, through the prostate, to the end of the penis. The accessory glands also secrete fluids, the seminal plasma, that mixes with sperm and form the semen or seminal fluid (Jones & Lopez, 2014).

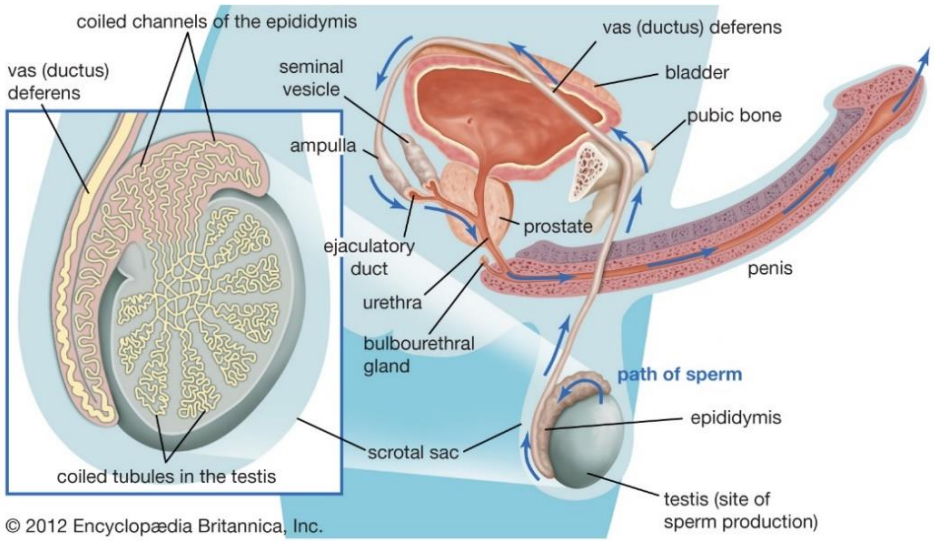


*Figure 5: The pathway that sperm follows from its production until ejaculation and transfer to the female genital tract.*

### *I.IV. Sperm-egg interaction and fertilization*

Even after ejaculation, it has been discovered that the male gamete is not capable of fertilization. Thus, further events in the female genital tract are required before fertilization occurs (Ikawa et al., 2010; Okabe, 2018).

The first event is called *capacitation*. Capacitation is essential to acquire the spermatozoon the capability to penetrate the zona pellucida, a thick vitelline envelope surrounding fully mature oocytes. It also prepares the sperm to undergo a process named acrosome reaction (Okabe, 2018; Stival et al., 2016). Capacitation is a process observed only in mammalian spermatozoa but it should be noted that capacitation-related events can also be observed in other species (Okabe, 2018). During capacitation, sperm acquires a different motility pattern of high energy that is called hyperactivation and it is associated with many molecular and physiological events (Georgadaki et al., 2016; Okabe, 2018). At first, sperm are exposed to a higher $HCO^{-}_{3}$ concentration after ejaculation and during its transfer to the female genital tract. After that, a key step for the fertilization process occurs and includes cholesterol removal from the sperm membrane to acceptors found in the uterus or fallopian tubes, with the most important of them being albumin. As a result, several biophysical changes in the plasma membrane are observed, such as the increase of the membrane fluidity. An increase in the cAMP concentration leads also to the activation of the cAMP-PKA pathway (Molina et al., 2018; Stival et al., 2016). Activation of PKA results in phosphorylation of sperm proteins and hyperpolarization of the plasma membrane also occurs. Finally, sperm intracellular pH increases and specific channels are activated leading to an increase of $Ca^{2+}$ concentration (Georgadaki et al., 2016; Molina et al., 2018). The time required for capacitation varies and is affected by factors found in the female environment (Okabe, 2018).

It has been proposed that the increase in the calcium influx occurring at the end of capacitation is the event that triggers the *acrosome reaction* in many spermatozoa simultaneously (Okabe, 2018). However, since then, factors inducing the acrosome reaction have been investigated extensively. Nowadays, research shows that the irreversible binding of spermatozoa to ZP3, a glycoprotein found in the extracellular matrix of zona pellucida, is the event triggering acrosome reaction (Anifandis et al., 2014; Georgadaki et al., 2016). The acrosome reaction is a process involving the exocytotic release of acrosomal enzymes by fusion of the acrosome membrane with the plasma membrane of the sperm (Hirohashi & Yanagimachi, 2018; Okabe, 2018). Many of the enzymes that are secreted may have a role in the fertilization process (Okabe, 2018). However, the most important of them seems to be hyaluronidase which hydrolyses the hyaluronic acid of the cumulus oophorous, the outer layer of cells that surround the oocyte (Anifandis et al., 2014). In contrast with capacitation, the acrosome reaction is a phenomenon observed in most animals (Hirohashi & Yanagimachi, 2018).

After the acrosome reaction, the zona pellucida is digested and fusion of sperm's membrane to egg's membrane follows. This interaction involves a region of the sperm plasma membrane that does not participate in the acrosome reaction and is called the equatorial region. The fusion is triggered by the binding between zona pellucida and sperm. Among molecules that are involved in the gamete binding are fertilin α, fertilin β, and cyritestin (or ADAM1, ADAM2, ADAM3). Other molecules involved are proteins CD9, CRISP1, and IZUMO (Georgadaki et al., 2016; Ikawa et al., 2010).

The final step is the *cortical reaction*, a process involving the release of cortical granules from the oocyte preventing polyspermy, the fusion of multiple spermatozoa with one oocyte (Anifandis et al., 2014; Georgadaki et al., 2016). Then, male's and female's pronuclei form the nucleus of the zygote (Georgadaki et al., 2016).

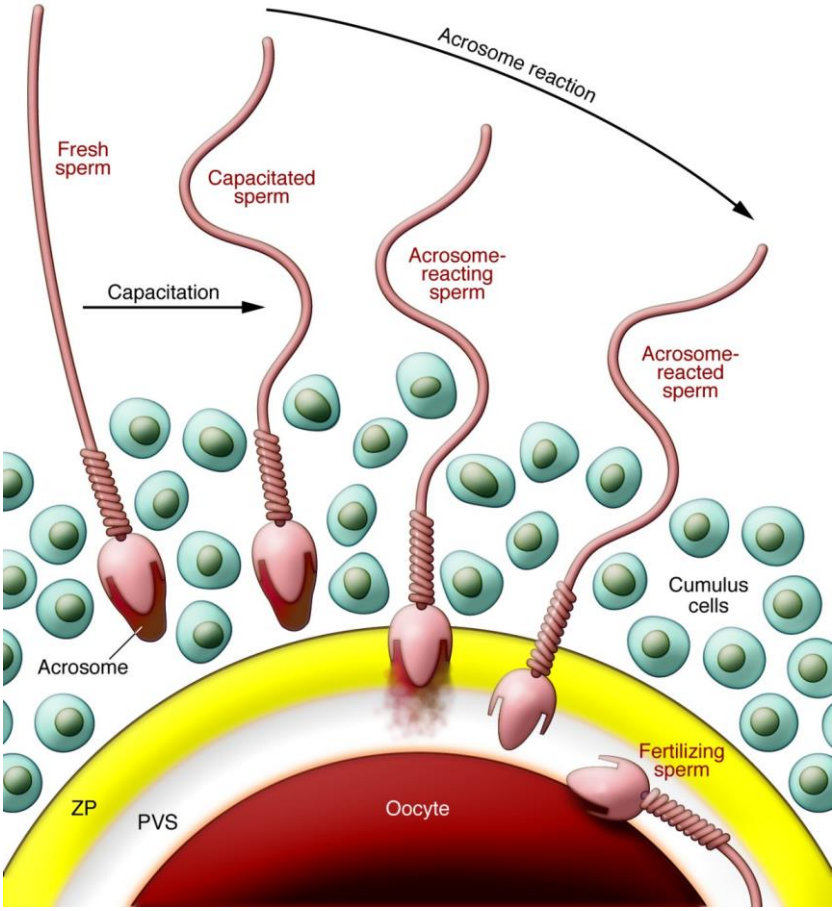


*Figure 6: The sequence of events during the fertilization process. ZP: Zona pellucida; PVS: Perivitelline space (Ikawa et al., 2010).*

# CHAPTER II: MALE INFERTILITY

Infertility is defined by *WHO* (World Health Organization) as "the inability to conceive after at least 12 months of regular, unprotected sexual intercourse" (Zegers-Hochschild et al., 2017). The term infertility sometimes is used interchangeably with the term *subfertility*. However, these terms should be differentiated by sterility, which is defined as the permanent inability to conceive, whereas infertility and subfertility refer to a restricted time period (Vander Borght & Wyns, 2018). Moreover, infertility can be classified as *primary* and *secondary*. Primary infertility is observed when a couple has never established a clinical pregnancy, whereas in secondary infertility the couple had at least one clinical pregnancy in the past (Benksim et al., 2018; Vander Borght & Wyns, 2018). Infertility is a global health problem as it is estimated that more than 186 million people worldwide are affected by infertility or 8-12% of reproductive-aged couples (Vander Borght & Wyns, 2018). Generally, the prevalence of infertility is on the rise and it has been reported that between 1990 and 2017 female infertility increased annually by 0.37% and male infertility increased annually by 0.29% (Sun et al., 2019).

Male infertility is the inability to conceive due to males. It can be described as a complex multifactorial condition with a wide range of phenotypic presentations including the complete absence of spermatozoa or changes in sperm quality and quantity (Krausz & Riera-Escamilla, 2018; Murshidi et al., 2020). The male factor is solely responsible in 20-30% of all cases and contributes with the female factor to 50% of cases overall (Agarwal et al., 2021; Vander Borght & Wyns, 2018). However, these numbers do not accurately represent cases of male infertility around the globe as there are many differences between different regions. More specifically, infertility rates are highest in Africa and Central/Eastern Europe (Agarwal et al., 2015).

Infertility can cause psychological problems, such as anxiety, fear, grief, and depression, as well as social problems (Agarwal et al., 2021; Karimi et al., 2015). Moreover, it can impose economic consequences for both patients and the healthcare system (Agarwal et al., 2021). Therefore, early diagnosis and treatments of infertility are extremely important.

## *II.I. Causes of Male Infertility and Risk Factors*

Male infertility is a complex disorder affected by many factors that interact and contribute to the phenotype. It can be caused by many different conditions, involving hormonal disorders, infections, illnesses, injuries, or anatomic abnormalities. Genetic factors also play an important role (Dimitriadis et al., 2017; Katz et al., 2017; Vander Borght & Wyns, 2018). Though it should be noted that in many cases it is difficult to identify the factors causing male infertility, and thus, 30-40% of male infertility cases are characterized as idiopathic (Katz et al., 2017).

More specifically, according to the mechanism of action, causes of male infertility can be classified into three categories: pre-testicular, testicular, and post-testicular (Katz et al., 2017).

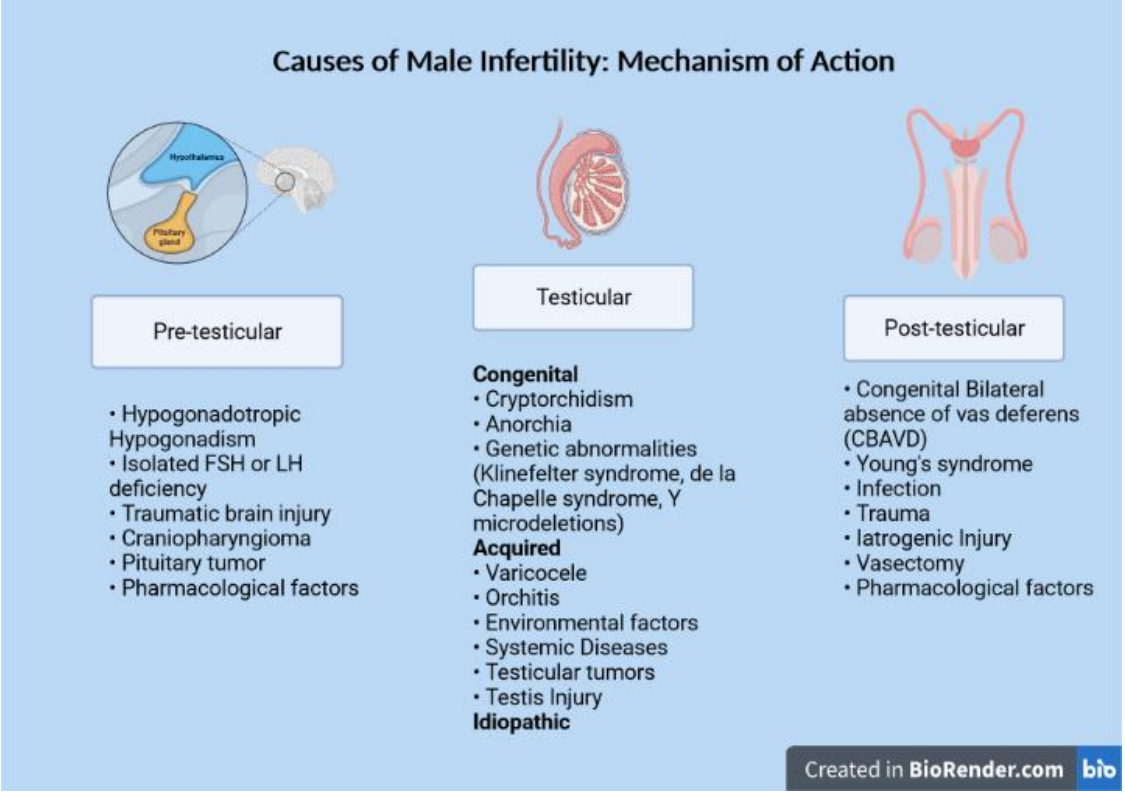


*Figure 7: The main causes of male infertility according to the mechanism of action. This figure was created by BioRender.com.*

*Pre-testicular causes* refer to abnormalities in the hypothalamus or/and the pituitary gland leading to abnormal hormone production. Thus, levels of various hormones, such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), or testosterone, can be decreased leading to several impairments e.g., in spermatogenesis (Dimitriadis et al., 2017; Katz et al., 2017). A pre-testicular cause is Hypogonadotropic hypogonadism that involves insufficient secretion of FSH and LH due to absent or insufficient secretion of gonadotropin-releasing hormone (GnRH) or a compromised pituitary. Thus, the testicular function is impaired, and the spermatogenesis process is affected. In some cases, hypogonadotropic hypogonadism can have a genetic origin and it is associated with mutations in *KAL-1* or other genes (Dimitriadis et al., 2017; Fraietta et al., 2013). Other pituitary diseases included in pre-testicular causes of male infertility are isolated LH or FSH deficiency, traumatic brain injury, craniopharyngioma, pituitary tumors (usually prolactinomas), etc. (Dimitriadis et al., 2017). Pharmacological factors can also lead to pre-testicular male infertility (Katz et al., 2017).

*Testicular causes* of male infertility include a wide range of factors that cause testicular dysfunction and thus, affect spermatogenesis and normal sperm production

(Dimitriadis et al., 2017; Vander Borght & Wyns, 2018). Testicular dysfunction can also be congenital, acquired, or idiopathic (Vander Borght & Wyns, 2018).

Congenital testicular failure can be due to several factors, including genetic abnormalities. More specifically, some of them are:

- **Cryptorchidism**: It is the most common genital problem and refers to the absence of at least one testicle from the scrotum. In most cases, the cause of cryptorchidism cannot be determined but it is considered that genetic, hormonal, and environmental factors are involved (Dimitriadis et al., 2017).
- **Anorchia**: It is a disorder of sex development that is characterized by the absence of testes in a 46, XY individual with a male phenotype (Vander Borght & Wyns, 2018). The cause of anorchia is still unknown (Mangaraj et al., 2017).
- **Genetic abnormalities** can also lead to male infertility as it is estimated that approximately 10% of the human genome is involved in reproduction (Choy & Eisenberg, 2018). The most common genetic abnormality detected in infertile men is *Klinefelter syndrome*, also known as 47, XXY (Choy & Eisenberg, 2018; Dimitriadis et al., 2017; Krausz & Riera-Escamilla, 2018). It is estimated that the prevalence of Klinefelter syndrome is 1 to 600 (Krausz & Riera-Escamilla, 2018) and affected males have smaller testes and decreased testosterone production. Other symptoms include reduced muscle mass, reduced body, and facial hair, or gynecomastia. Klinefelter syndrome is the most common genetic cause of azoospermia, which is defined as the complete absence of spermatozoa. Except for the 47, XXY genotype, patients can also be mosaics (46, XY and 47, XXY) (Choy & Eisenberg, 2018; Dimitriadis et al., 2017; Krausz & Riera-Escamilla, 2018).
  *46, XX male syndrome* or also characterized as *de la Chapelle syndrome* is a rare syndrome observed with a frequency of 1 to 20,000 children. It is mainly caused by a translocation of the *SRY* gene, that directs the sex-determination pathway towards male development, on the X chromosome leading to azoospermia and other clinical characteristics that range from typical to ambiguous genitalia (Dimitriadis et al., 2017; Krausz & Riera-Escamilla, 2018).
  *Y chromosome microdeletions* have also been associated with altered sperm parameters. More specifically, microdeletions in the long arm of the Y chromosome (Yq) are the most common genetic cause of impaired sperm production and it is estimated that they are found in 5-10% of infertile men (Vander Borght & Wyns, 2018). Many years ago, it was discovered that in the long arm of the Y chromosome are located many genes that are essential for spermatogenesis. This region (*Figure 8*) is called azoospermia factor region (AZF) and it can be divided into three subregions: AZFa, AZFb, and AZFc (Krausz & Riera-Escamilla, 2018). AZFc microdeletions are most common, whereas AZF deletions are most usually associated with azoospermia (Dimitriadis et al., 2017).

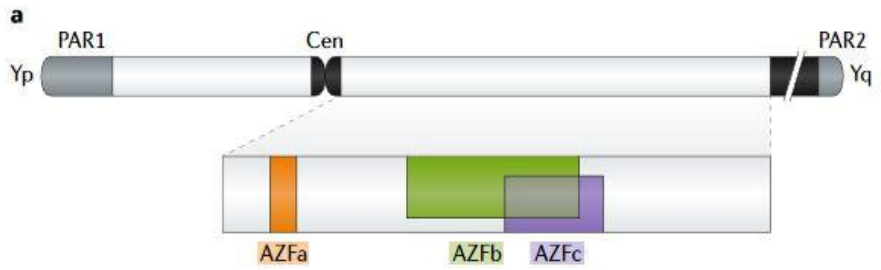


*Figure 8: The azoospermia factor region (AZF) and its subregions. Cen, centromere; PAR, pseudoautosomal regions (Krausz & Riera-Escamilla, 2018).*

Regarding acquired testicular failure, the most common causes are endogenous or exogenous factors, trauma, etc. (Vander Borght & Wyns, 2018). Some of the most important are:

- **Varicocele**: It is a term used to describe the abnormal enlargement of a specific type of veins, pampiniform plexus, found in the scrotum (Dimitriadis et al., 2017; Miyaoka & Esteves, 2012). Its frequency is generally high as it can be present in 4% to 23% of males. However, the incidence is even higher in males with fertility problems (Miyaoka & Esteves, 2012). Thus, varicocele is associated with male infertility, but the exact mechanism is not fully understood. Oxidative stress, an increase in testicular temperature, and hypoxia seem to play an important role (Vander Borght & Wyns, 2018).
- **Orchitis**: Orchitis involves the inflammation of the testicle, usually caused by bacteria or viruses (Azmat & Vaitla, 2021; Dimitriadis et al., 2017). More specifically, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus,* and *Streptococcus* species are the most common bacteria causing orchitis, whereas the most common viruses are coxsackievirus, varicella, and dengue virus (Azmat & Vaitla, 2021).
- **Environmental factors**: Some substances found in the environment are gonadotoxic and thus, interfere with spermatogenesis and affect sperm quality. They may act directly on germ cells or indirectly through alterations in the endocrine system. For example, electromagnetic radiation transmitted by mobile phones or other devices can affect sperm quality, maybe due to its heating effect (Dimitriadis et al., 2017; Katz et al., 2017). Prescription drugs and medications, such as antihypertensive agents, antibiotics, chemotherapy drugs, or recreational drugs, can also affect sperm parameters and fertilization capacity. Other examples include exposure to heavy metals, pesticides, or tobacco smoking (Dimitriadis et al., 2017).
- **Systemic Diseases**: Several systemic diseases have been associated with male infertility. Some of them are chronic renal failure that can affect the testicular function and hormone levels (e.g., testosterone), liver failure, or some hematological diseases (Dimitriadis et al., 2017).
- **Testicular tumors**: Testicular cancer is the most common type of cancer among men of reproductive age. Sperm quality is usually affected in patients but

treatment of cancer with chemotherapy or radiation can also cause DNA damage in sperm or other problems such as oligozoospermia or azoospermia (Ping et al., 2014).

- **Testis Injury**: Testicular trauma by accident, after surgery, work injury, or sports activities is a very common cause of male infertility. The effect of trauma on sperm parameters can vary (Dimitriadis et al., 2017).

Finally, *post-testicular causes* of infertility are due to obstruction or ejaculatory dysfunction. The obstruction can be found in the epididymis, the vas deferens, or the ejaculatory duct. Acquired causes of obstruction include infections, trauma, or iatrogenic injuries (Dimitriadis et al., 2017; Vander Borght & Wyns, 2018). Among the most common causes of obstruction, congenital bilateral absence of vas deferens (CBAVD) is usually found in men with cystic fibrosis (CF). Cystic fibrosis is an autosomal recessive disease that causes severe damage to the lungs, the digestive system, and other organs in the human body. Most of the patients carry a mutation in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene that is called ΔF508 and causes loss of an amino acid at the 508$^{th}$ position of the protein. Most of them are also diagnosed with obstructive azoospermia (Hui Chen et al., 2012). Other post-testicular causes of male infertility are Young's syndrome, nerve injury, vasectomy, iatrogenic injury to the vas deferens, or other pharmacological factors (Katz et al., 2017).

### *II.II. Diagnosis and Evaluation of Male Infertility*

Evaluation of fertility is proposed for couples unable to conceive after at least 12 months of regular, unprotected sexual intercourse but when the female is older than 35 years old the evaluation is proposed after only 6 months (Agarwal et al., 2021). The evaluation and successful diagnosis of infertility is a challenging process as many factors are involved, as it has already been described. The basic process of fertility evaluation includes physical examination, taking of the medical history, and semen analysis. Further evaluation includes hormonal assessment and genetic testing (Agarwal et al., 2021; Barratt et al., 2017; Katz et al., 2017).

The taking of medical history involves several different aspects, from past illnesses and medications to exposure to environmental factors and couple's sexual practices (Agarwal et al., 2021; Barratt et al., 2017; Katz et al., 2017). Any diseases, as well as childhood conditions (e.g., cryptorchidism, testicular torsion, or trauma, etc.) and infections of the male urogenital tract, such as prostatitis, urethritis, epididymitis, etc., should be reported in the clinical doctor (Agarwal et al., 2021; Barratt et al., 2017) as male infertility is associated with chronic inflammation (Azenabor et al., 2015). Infection by *E. coli*, *Chlamydia trachomatis*, and other microorganisms are also present in a large part of infertile men (Agarwal et al., 2021). Information about past surgical interventions (orchidopexy, pelvic surgery, vasectomy, etc.) or malignancies should also be provided to the doctor. Regarding lifestyle factors, research shows that alcohol consumption, smoking, use of drugs for recreation (cocaine, cannabis, opioid narcotics, etc.), use of supplements (vitamins, antioxidants, etc.), use of anabolic steroids, and obesity have been associated with male infertility (Agarwal et al., 2021;

Barratt et al., 2017). The doctor should also take a sexual history that includes factors such as libido, the frequency of sexual intercourse, erectile and ejaculatory function (Agarwal et al., 2021). Finally, the family history is very important, too. Other members of the family may suffer from infertility, but cases of cystic fibrosis and androgen receptor deficiency should also be investigated as they are associated with male infertility (Agarwal et al., 2021; Barratt et al., 2017).

Physical examination is also a critical step for the diagnosis of male infertility. It includes an assessment of body habitus, an assessment of secondary sexual characteristics, and genital examination (Agarwal et al., 2021; Barratt et al., 2017; Katz et al., 2017). Reduced muscular development, decreased body hair, obesity or gynecomastia can be characteristics of endocrinopathies, or other diseases associated with male infertility (Agarwal et al., 2021). Moreover, the genital examination should include examination of the phallus, the testicles (location, size, consistency), the epididymis (enlargement, hypoplastic epididymis), the prostate (size, consistency), and the spermatic cords to detect a varicocele or a hydrocele (Barratt et al., 2017).

Semen analysis is the most critical step for the diagnosis of male infertility (Agarwal et al., 2021; Barratt et al., 2017; Katz et al., 2017; Miller & Vukina, 2020). Standard semen analysis involves the evaluation of microscopic and macroscopic parameters, as presented in *Figure 9* (Agarwal et al., 2021). According to *WHO* reference values released in 2010, the normal values for sperm parameters are presented in *Table 1*.

*Table 1: Normal values for semen parameters according to WHO (2010)*

| Semen Analysis | |
|---|---|
| **Volume** | ≥ 1.5 mL |
| **Sperm Concentration** | ≥15×$10^6$/mL |
| **Total sperm count** | ≥39×$10^6$/mL |
| **Sperm motility (%)** | ≥32% |
| **Sperm vitality (%)** | ≥58% |
| **Sperm morphology (% normal)** | ≥4% |

Semen analysis contains the evaluation of parameters associated with sperm count, morphology, and motility, measuring the overall testicular health, spermatogenesis, spermiation, and other functional properties (Agarwal et al., 2021; Barratt et al., 2017; Miller & Vukina, 2020). According to the results, semen can be classified into specific types associated with quantitative or qualitative disturbances. More specifically, *azoospermia* is defined as the absence of spermatozoa in the ejaculate and *cryptozoospermia* is characterized by very low sperm concentration (< 1 million sperm/ml), whereas *oligozoospermia* refers to a concentration lower than 15 million

sperm/ml (Agarwal et al., 2021; Krausz & Riera-Escamilla, 2018). Moreover, *asthenozoospermia* is the medical term used for reduced sperm motility, below the reference limit, and *teratozoospermia* is defined as a percentage of morphologically normal spermatozoa below the reference limit (Agarwal et al., 2021).

Except for semen analysis, hormonal assessment can also be used for the diagnosis of male infertility (Agarwal et al., 2021; Barratt et al., 2017). The recommended hormonal evaluation includes the measurement of follicle-stimulating hormone and total testosterone. If the levels of total testosterone are low, a further hormonal evaluation is required including for example measurement of luteinizing hormone, free testosterone, or prolactin (Agarwal et al., 2021; Katz et al., 2017).

Genetic testing is another way to evaluate the status of male fertility as genetic abnormalities account for a large percentage of male infertility (Agarwal et al., 2021; Barratt et al., 2017; Katz et al., 2017; Miller & Vukina, 2020). Genetic testing can include karyotyping for the identification of structural or numerical chromosomal defects, analysis for Y chromosome microdeletion, and *CFTR* mutations (Barratt et al., 2017; Katz et al., 2017).

It should also be noted that specialized tests (*Figure 9*) can also be required when specific clinical indications are present e.g., after a failure of in-vitro fertilization (Agarwal et al., 2021). Sperm DNA fragmentation is a term referring to sperm DNA damage that can be due to apoptosis during spermatogenesis, defects in the spermiogenesis process, or exposure to environmental toxicants (Miller & Vukina, 2020; Sakkas & Alvarez, 2010). DNA damage can also be caused by oxidative stress; thus, measurement of reactive oxygen species can also be performed. Mitochondrial function can also be assessed. Assays evaluating sperm-zona pellucida binding or acrosome function are no longer used (Agarwal et al., 2021).

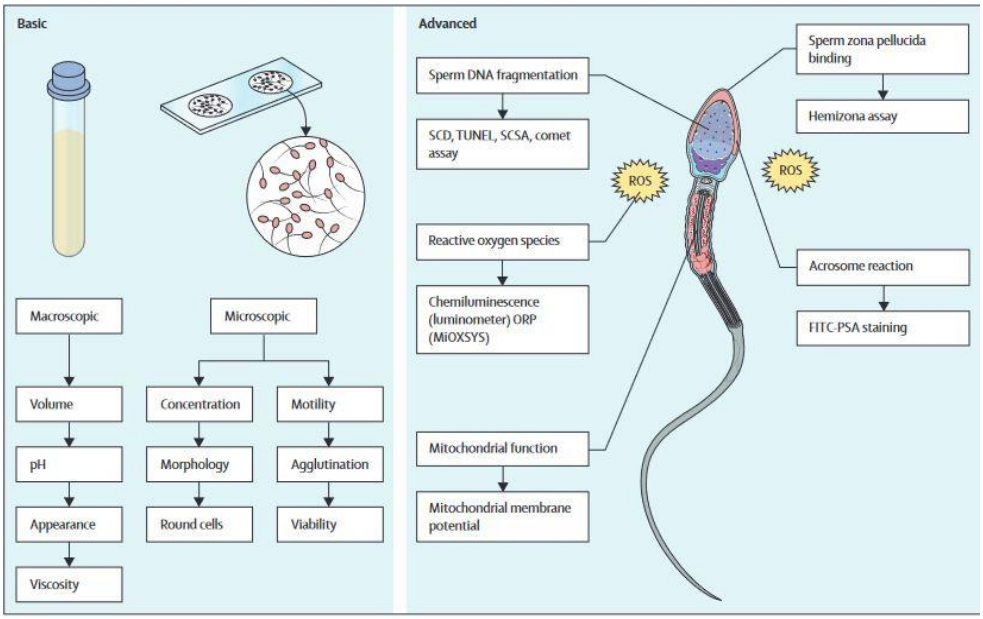


*Figure 9: Laboratory assessment of male infertility. Semen analysis is considered the most critical step and includes the evaluation of microscopic and macroscopic parameters. Specialized tests include*

*determination of ROS, sperm DNA fragmentation assay, acrosome reaction, sperm-zona pellucida binding, and mitochondrial function with different techniques presented on boxes* (Agarwal et al., 2021).

### *II.III. Management of Male Infertility*

Although recently there have been several advances in the field of male infertility, its management is still limited. More specifically, the latest manual of WHO for management of male infertility was published two decades ago, in 2000 (Karavolos et al., 2020). Since then, assisted reproductive technologies remain the mainstay of treatment of male infertility but there are also some other approaches often used to treat male infertility and a lot of research is conducted for potential therapeutic options. Thus, the most common approaches for the management of male infertility are the following:

- Lifestyle changes: Although there is limited evidence that specific lifestyle changes improve male infertility, environmental factors have been associated with male infertility. According to these risk factors, a change of lifestyle and some daily habits can offer a therapeutic opportunity. It is generally recommended for patients to avoid smoking and use of marijuana or other recreational drugs, avoid alcohol consumption and maintain a healthy weight by eating a balanced diet rich in fresh fruits and vegetables, fish, poultry, and cereals. The intake of specific vitamins and minerals may also improve semen quality. Among them are vitamin B12, vitamin C, vitamin E, and zinc (Pourmoghadam et al., 2018). Antioxidant supplements are also recommended (Karavolos et al., 2020; Pourmoghadam et al., 2018).
- Medical: In a small percentage of cases, male infertility is due to hormonal abnormalities that can be treated with medicines. More specifically, in men with hypogonadotropic hypogonadism, clomiphene citrate can be administered orally to increase androgen production and spermatogenesis (Pan et al., 2018), but therapy with gonadotropin-releasing hormone can also be used (Pourmoghadam et al., 2018).
- Surgery: Male infertility can be treated by surgery also, only on specific causes. For example, varicocele can be corrected by different surgical approaches, such as laparoscopic, microsurgical, etc. Testicular cysts and any obstruction in the ductal system that cause male infertility can also be managed surgically (Pan et al., 2018; Pourmoghadam et al., 2018).
- Assisted Reproductive Technology (ART): In the last decades, developments in the field of ART have been remarkable (Szamatowicz, 2016). In vitro fertilization (IVF) was the most popular technique used for infertility problems for many years. IVF comprises a complex series of procedures for successful fertilization. It involves retrieval of mature oocytes and sperm cells that are co-incubated and then the fertilized embryos are transferred to the uterus (Pan et al., 2018; Pourmoghadam et al., 2018). However, the introduction of intracytoplasmic sperm injection (ICSI) has revolutionized the management of male infertility. ICSI also involves retrieval of mature oocytes, but only a single

sperm is used and is injected into the center of the egg by a micropipette (Khorram et al., 2001; Pan et al., 2018). ICSI is usually used in cases of low sperm count and even in cases of azoospermia or cryptozoospermia (Pan et al., 2018) because apart from ejaculated spermatozoa, epididymal or testicular spermatozoa can be used successfully for ICSI. Thus, several surgical retrieval techniques have been developed and they are used in everyday infertility practice for ICSI (Khorram et al., 2001).

Among the limitations of ART, the increased cost is an important consideration for couples, as well as the fact that it requires a considerable commitment of time and energy (Pourmoghadam et al., 2018). Many of the techniques used are also associated with health risks (Pan et al., 2018; Pourmoghadam et al., 2018; Szamatowicz, 2016). It should be noted, however, that these techniques are not a treatment per se, but they are used to achieve a successful pregnancy (Karavolos et al., 2020).

- Stem cell therapy: In the last decade, a lot of research is also conducted regarding stem cells as they have a potential therapeutic value for treating male infertility. More specifically, spermatogonial stem cells (SSC) are undifferentiated cells that are essential for spermatogenesis, and they are also characterized by two unique properties: self-renewal and differentiation. In one possible approach, SSCs are harvested from the testis and after that cultured and differentiated into fully mature spermatozoa. Although SSCs show promise for future applications in clinical practice, there are several limitations including their low concentration in the testis, difficulties to isolate and distinguish them from other cells in the testes as well as culturing process (Forbes et al., 2018; Pourmoghadam et al., 2018).

# CHAPTER III: SPERM AND ENERGY METABOLISM

## *III.I. Energy Production in Spermatozoa*

Adenosine Triphosphate (ATP) is an energy storage molecule and is usually called the "energy currency" of the cell due to the high energy bonds that link the phosphate groups (Bonora et al., 2012). Energy is also essential to support sperm function. More specifically, mammalian spermatozoa use ATP for movement and processes required for fertilization, such as capacitation, hyperactivation, and the acrosome reaction (Du Plessis et al., 2015; Misro & Ramya, 2012; Visconti, 2012). ATP is also involved in many regulatory signaling pathways, thus, it is an extremely important molecule for sperm as it provides all the energy to drive and support sperm's functions (Visconti, 2012).

As the site of sperm deposition is far from the site of fertilization, spermatozoa should be provided with an adequate quantity of energy to support their motility and transport. Moreover, as it has already been described, spermatozoa must undergo a maturation process in the female tract, capacitation, which is characterized by hyperactivation. Therefore, many energy-dependent processes should occur for successful fertilization (Du Plessis et al., 2015; Gibb & Aitken, 2017). An extra challenge for sperm bioenergetics is the fact that during spermatogenesis the spermatozoa undergo an extensive remodeling that involves their transcriptional inactiveness and removal of the cytoplasm. As a result, spermatozoa do not have enough intracellular space to store energy reserves (Gibb & Aitken, 2017; Tourmente et al., 2015). Spermatozoa also face differences in oxygen and metabolic substrates availability as they move through the female tract (Tourmente et al., 2015).

To overcome the limitations stated above, two metabolic pathways are mainly used by mammalian sperm for ATP production: Glycolysis and Oxidative Phosphorylation (OXPHOS). The two metabolic pathways are compartmentalized. More specifically, the midpiece concentrates all mitochondria required for oxidative phosphorylation while in the principal piece of the flagellum all the enzymes required for glycolysis are found (Misro & Ramya, 2012; C. Mukai & Travis, 2012; Tourmente et al., 2015; Visconti, 2012). Glycolysis also occurs in the head, according to research (Du Plessis et al., 2015). It should also be noted that this restricted localization of mitochondria in sperm is a highly conserved characteristic observed from external fertilizers, such as sea urchins, to mammals (C. Mukai & Travis, 2012).

## *III.II. Glycolysis*

Glycolysis is a metabolic pathway that can be performed in the absence of oxygen; thus, it is called an anaerobic process (Berg et al., 2012), that in spermatozoa takes place in the head and the principal piece of the flagellum (Du Plessis et al., 2015). Large polar molecules cannot diffuse across membranes. Therefore, glucose transporters are membrane-bound proteins that facilitate substrates to pass through the plasma membrane of the flagellum and to be used for glycolysis (Misro & Ramya, 2012; Navale & Paranjape, 2016).

During glycolysis, glucose, $C_6H_{12}O_6$, is oxidized to pyruvate with a series of enzyme-catalyzed reactions that lead to a final yield of two ATP molecules for every molecule of glucose that is oxidized. The glycolytic pathway can be divided into three main stages (*Figure 10*). In *Stage 1* glucose is trapped in the cell and is destabilized by its conversion into fructose 1,6-bisphosphate. The next stage, *Stage 2*, involves the cleavage of the fructose 1,6-bisphosphate into two three-carbon molecules. In *Stage 3*, these molecules are oxidized, and ATP is produced. After that, for the continuation of glycolysis, pyruvate is used for NAD+ regeneration from NADH that is also produced during glycolysis. More specifically, in the next steps, pyruvate is further oxidized, and the carboxyl group is lost to yield the acetyl group of acetyl-coenzyme A. The acetyl group is then oxidized completely to $CO_2$ by the citric acid cycle (Berg et al., 2012).

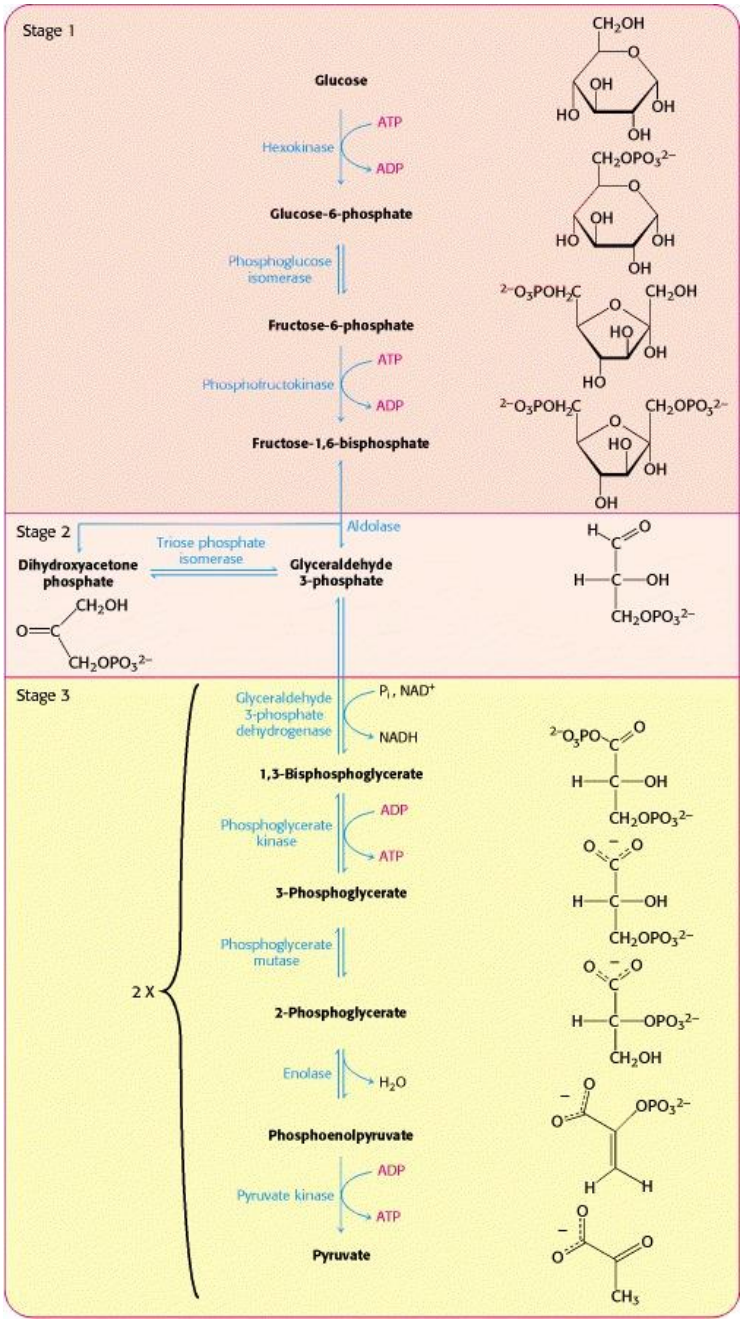


*Figure 10: Glycolysis pathway and stages* (Berg et al., 2012).

Several of the glycolytic enzymes have been identified mainly in the fibrous sheath of the principal piece in mammals (Du Plessis et al., 2015; C. Mukai & Travis, 2012), but it should also be noted that in spermatozoa most of the steps described above are catalyzed by sperm-specific isoforms. Some characteristic examples are the spermatogenic glyceraldehyde phosphate dehydrogenase (GAPDHS), lactate dehydrogenase C (LDHC), and phosphoglycerate kinase 2 (PGK2) (Du Plessis et al., 2015; Tourmente et al., 2015; Visconti, 2012). Until today, it has been observed that for at least 6 of the 10 glycolytic enzymes there are germ cell-specific modifications (C. Mukai & Travis, 2012).

### *III.III. Oxidative Phosphorylation (OXPHOS)*

Mitochondria are usually called the "powerhouse of the cell" as they play a major role in the most efficient process of ATP production, oxidative phosphorylation. A mature spermatozoon contains approximately 50-75 mitochondria which provide it with sufficient energy for several processes, such as the acrosome reaction (Barbagallo et al., 2020). Sperm mitochondria also differ morphologically and functionally from that found in somatic cells and possess sperm-specific isoforms of proteins and isoenzymes, such as a testis-specific subunit of the cytochrome c oxidase (COX6B2) (Barbagallo et al., 2020; Du Plessis et al., 2015).

Oxidative phosphorylation is a complex process that involves two components of the inner mitochondrial membrane: the respiratory chain and ATP synthase (Amaral et al., 2013; Du Plessis et al., 2015; Misro & Ramya, 2012). More specifically, as presented in *Figure 11*, the oxidative phosphorylation system (OXPHOS) of the mitochondrial inner membrane is comprised of five enzymes (complexes I-V): NADH: ubiquinone reductase (Complex I), succinate dehydrogenase (Complex II), quinol-cytochrome c reductase (Complex III), cytochrome c oxidase (Complex IV) and H + -transporting two-sector ATPase or FoF1-ATPase (Complex V), which synthesizes ATP. In human, all the enzymes are multimeric, and, except for Complex II, they have subunits encoded both in the mitochondrial genome (mtDNA) and the nuclear genome (nDNA) (Signes & Fernandez-Vizarra, 2018). It has been found that 92 structural proteins are needed to build up OXPHOS complexes (Koopman et al., 2013).

Through a series of redox reactions catalyzed by Complexes I-IV, NADH is used to donate electrons to oxygen. More specifically, for every pair of electrons transferred to $O_2$, 10 $H^+$ are pumped out through the inner mitochondrial membrane. The $H^+$ gradient that is formed favors the reuptake of $H^+$ through the ATP synthase complex and as a result ATP generation is observed (Berg et al., 2012; Visconti, 2012). For every molecule of glucose that is oxidized, thirty molecules of ATP are produced. Thus, OXPHOS is a more efficient pathway for energy generation but at the same time, it leads to the production of significant quantities of reactive oxygen species (ROS) as a by-product (Amaral et al., 2013; Du Plessis et al., 2015; Misro & Ramya, 2012).

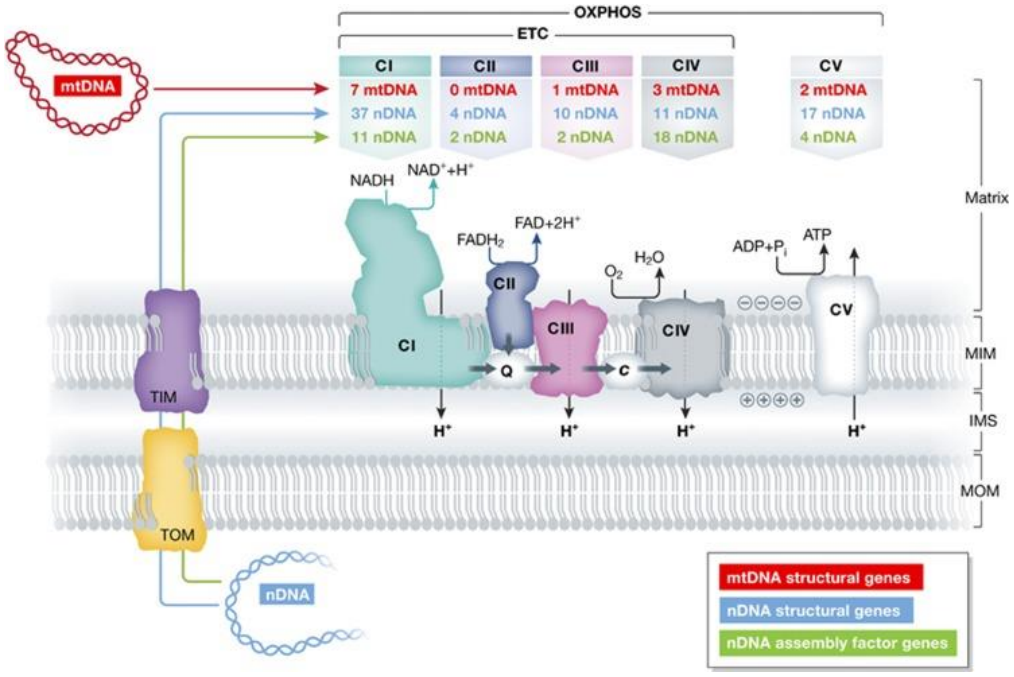


*Figure 11: Complexes of mitochondrial oxidative phosphorylation (OXPHOS) and their genetic origin* (Koopman et al., 2013).

### *III.IV. What fuel do spermatozoa use?*

It is well established that ATP is essential for successful fertilization. According to research, in the absence of metabolic substrates, the sperm becomes immotile and unable to hyperactivate as the ATP is consumed and no more can be produced. Thus, fertilization cannot occur (Visconti, 2012).

However, the question that arises is which is the main biochemical pathway that provides the energy for sperm motility, glycolysis, or oxidative phosphorylation? Though the findings are contradicting, it is widely believed that ATP produced from the two different pathways plays different roles (*Figure 12*) (Barbagallo et al., 2020; Du Plessis et al., 2015; Visconti, 2012).

At first, flagellum movement is required for sperm motility and movement through the female genital tract until reaching the oocyte. The dynein ATPases are associated with the axoneme and use the chemical energy of ATP hydrolysis for the flagellar beating of spermatozoa. Thus, as the main consumers of ATP are the dynein ATPases, a question is how efficient it is to transfer the energy produced by mitochondria all this way down to flagellum (Du Plessis et al., 2015; C. Mukai & Travis, 2012). The results are conflicting, but most researchers propose that the local ATP production to the site of utilization, meaning the flagellum, is more efficient. Thus, the presence of glycolytic enzymes in the tail suggests that the glycolysis pathway is the main source of ATP for the flagellar beat and sperm motility (Du Plessis et al., 2015).

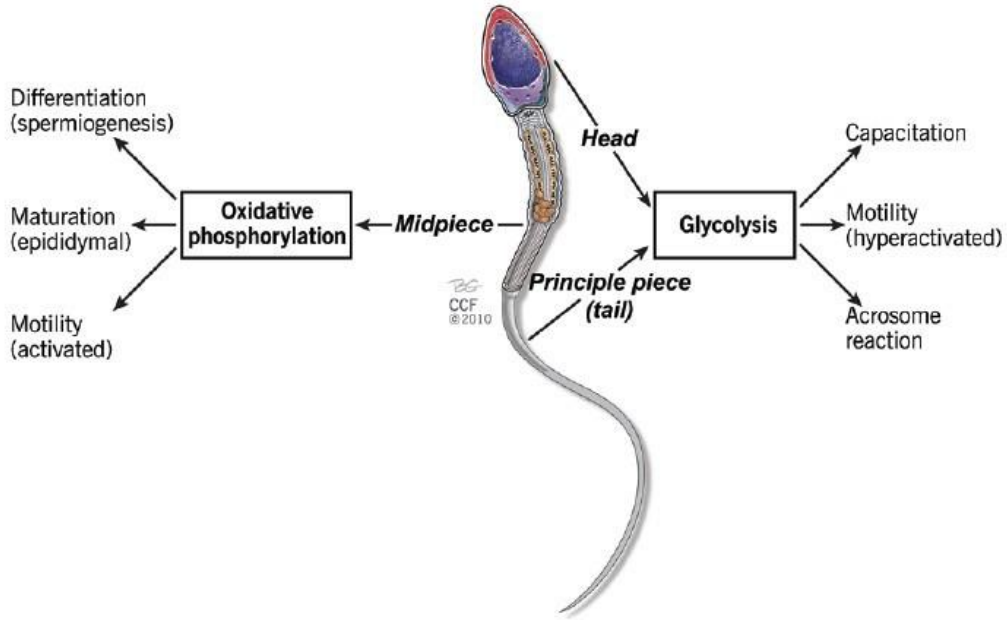


*Figure 12: A representation of compartmentalization regarding energy production in sperm and sources of energy for different processes and functions of human sperm* (Du Plessis et al., 2015).

The functional importance of the ATP produced by the glycolysis pathway is proved by several experiments. More specifically, experiments in mice show that the glycolysis pathway is essential for male fertility as knock-out mice for GAPDHS and PGK2 are infertile due to reduced sperm motility (Du Plessis et al., 2015; C. Mukai & Travis, 2012; Visconti, 2012). In another experiment, a glucose analog was used to inhibit glycolysis in mice sperm and as a result, a decrease in motility was observed (Chinatsu Mukai & Okuno, 2004). In contrast, inhibition of mitochondrial function did not affect sperm motility or ATP levels (Gibb & Aitken, 2017; C. Mukai & Travis, 2012). However, OXPHOS activation for ATP production to support sperm motility may require specific conditions as Zhu et al., (2019) had recently shown that mitochondrial oxidative phosphorylation is activated to produce ATP when low glucose levels are observed.

In conclusion, glycolysis seems to be the preferred pathway for human spermatozoa, but OXPHOS energy is required for other processes, such as differentiation and maturation (Du Plessis et al., 2015). More specifically, mitochondria play a key role in events required for fertilization, meaning the capacitation and the acrosome reaction. To better understand the role of mitochondria during capacitation, scientists used sperm samples before and after swim-up treatment. They found that mitochondrial respiratory activity in the cells incubated under capacitating conditions was significantly higher than that in the cells before swim-up. Thus, ATP generation through OXPHOS is highly observed during capacitation (Stendardi et al., 2011). Mitochondria are also required in capacitation-dependent tyrosine phosphorylation in mammalian spermatozoa and the mitochondrial apoptotic pathway might prevent DNA-damaged sperm from participating in fertilization (Amaral et al., 2013; Du Plessis et al., 2015). A potential reason that explains the fact that OXPHOS is not the preferred

pathway for sperm motility, even if it is more efficient, is the extensive ROS production that accompanies ATP generation. It has been stated that ROS production can negatively affect fertility due to an increase in lipid peroxidation and DNA damage (Barbagallo et al., 2020; Gibb & Aitken, 2017).

All these suggest that both pathways are important for sperm motility and play different roles in order to achieve successful fertilization. Moreover, maybe the availability of substrates affects the pathway that is activated (Barbagallo et al., 2020; Du Plessis et al., 2015).

Studies in other animals also conclude that there are species-specific differences regarding the role of the two metabolic pathways on fertility (Du Plessis et al., 2015; Misro & Ramya, 2012; Visconti, 2012). For example, glycolysis seems to be preferred by species such as the human and mouse, but in horses inhibition of mitochondria function leads to a dramatic decrease of spermatozoa motility and a significant decrease of ATP levels (Gibb & Aitken, 2017). Moreover, glycolysis is essential for fertilization in mice, rats, and humans but not in bovines (Du Plessis et al., 2015).

### *III.V. Energy Metabolism and Male infertility*

Regarding male infertility, though it is challenging to decipher the relative importance of glycolysis and OXPHOS to sperm function, mutations in many genes in both pathways have been associated with fertility problems (Gibb & Aitken, 2017). In general, energy metabolism and its dysregulation seem to play an important role in asthenozoospermia (Asghari et al., 2017; Gibb & Aitken, 2017).

About glycolysis and male infertility, as it has already been described, knockout of a gene encoding for a sperm-specific glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase-S, leads to decreased ATP levels and decreased sperm motility (Du Plessis et al., 2015; Miki et al., 2004; C. Mukai & Travis, 2012). Similarly, disruption of PGK2, an isoenzyme that catalyzes a step required for ATP generation during glycolysis, causes a decrease in ATP levels and sperm motility (Danshina et al., 2010). Furthermore, recently it has been discovered that miRNAs that affect the glycolytic pathway can be associated with male infertility and in particular with asthenozoospermia (Zhou et al., 2018).

As it has already been stated, the OXPHOS pathway is not the main source of energy for sperm movement but defects in mitochondria have been associated with decreased motility (Amaral et al., 2013). More specifically, lower activities of Complexes I, II, and IV are observed in patients with asthenozoospermia in comparison with men without fertility problems (Barbagallo et al., 2020). Interestingly, mutations in OXPHOS genes have been associated with male infertility, and particularly mtDNA mutations and asthenozoospermia have raised scientist's attention (Holyoake et al., 2001; Shamsi et al., 2008). Single Nucleotide Polymorphisms (SNPs) in nuclear-encoded OXPHOS genes have also been associated with impairment of sperm production and oligozoospermia (Bonache et al., 2007). Finally, according to prediction models, the knockout of genes related to Complex III, IV, and V of OXPHOS

can lead to a reduction in ATP levels and subsequent fertility problems (Asghari et al., 2017).

Therefore, both Glycolysis and OXPHOS pathways play an important role in fertilization but the association between specific genes and male infertility still needs further exploration to provide a better understanding of the molecular mechanism.

## CHAPTER IV: THESIS OBJECTIVES

The aim of this study is to shed light on energy metabolism and male infertility. This can be achieved by Genome-Wide Association Study (GWAS) for detection of genetic variants (SNPs) significantly related to male infertility, or specific subtypes of male infertility (oligozoospermia, asthenozoospermia, teratozoospermia), in Glycolysis and OXPHOS genes by comparing normozoospermic (control group) and non-normozoospermic individuals (case group) or by comparing individuals of different subfertility status (oligozoospermia, asthenozoospermia or teratozoospermia) and normozoospermic individuals. Whole Genome Sequencing of pooled samples characterized as normozoospermic or non-normozoospermic (asthenozoospermic, oligozoospermic, teratozoospermic) can also help to detect and characterize more variants, and especially novel, that are found in Glycolysis and OXPHOS genes.

Identifying genomic regions responsible for genetic variation in male infertility will enhance the understanding of the role of the two most important pathways for energy generation, Glycolysis and OXPHOS, in male infertility and contribute to a better understanding of the patient's profile and phenotype's subcategory. This may also point to opportunities for new biomarkers and clinical diagnosis of male infertility.

# Materials and Methods

As the aim of this thesis was to study polymorphisms in glycolysis and OXPHOS genes, two different types of analyses were performed. Thus, the study consists of two parts. In Part A, Genome-Wide Association Study (GWAS) was conducted, while in Part B, Whole Genome Sequencing Analysis (WGS) was used.

## A. GENOME-WIDE ASSOCIATION STUDY (GWAS)

A Genome-wide association study is an approach widely used in genetics research to associate specific genetic variations, Single Nucleotide Polymorphisms (SNPs), with a particular disease, in this case, male infertility. GWAS are based on SNP arrays which are used for the analysis of common variants. In brief, as presented in *Figure 13*, DNA samples from well-phenotyped individuals are collected and after that, SNP arrays are used for the identification of the genotype of each sample (Krausz & Riera-Escamilla, 2018; Uitterlinden, 2016). Commercial genotyping arrays can genotype thousands of SNPs simultaneously with high accuracy. Data Analysis follows to identify SNPs associated with the phenotype of interest using statistical tests (Uitterlinden, 2016).

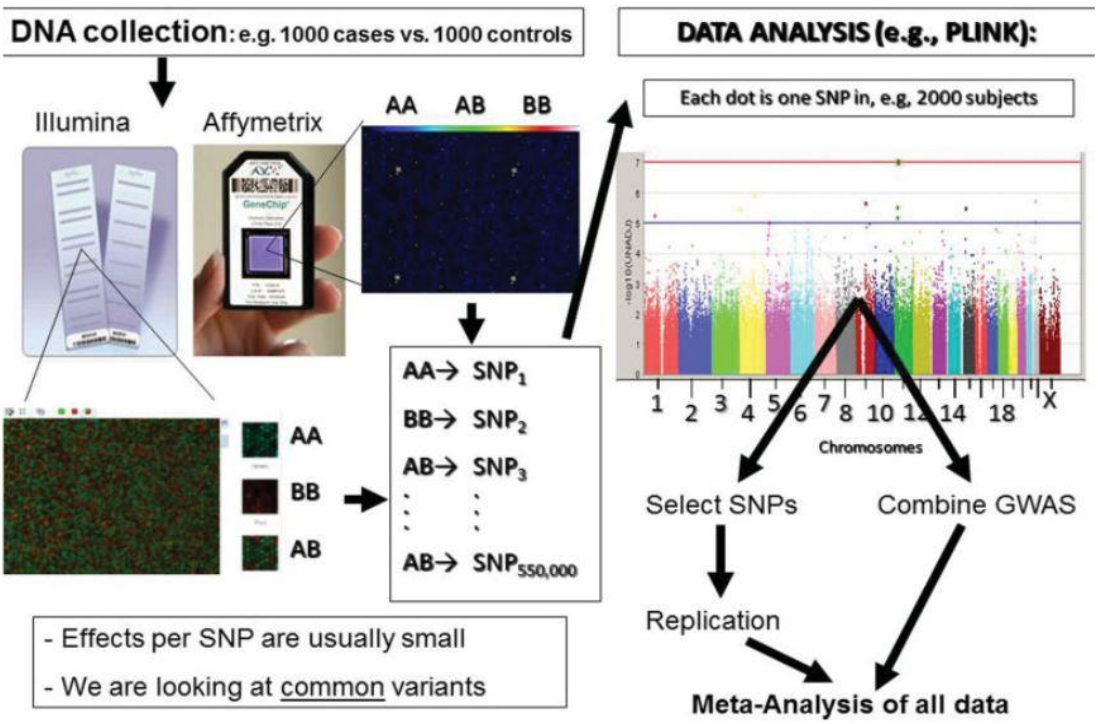


*Figure 13: Representation of the most important steps on a typical GWAS analysis.*

### *Patient Selection and Biological Material*

In this study, 576 semen samples were collected in cooperation with the "Embryolab Fertility Clinic" (Thessaloniki, Greece). They were derived from individuals aged between 18-40 years who have given approved informed consent. As this study is also limited to the Greek population, a questionnaire was given before to individuals to validate place of birth and other relevant information. Semen analysis was performed for all samples to obtain information about the phenotype.

According to the results of the semen analysis, all the samples were divided into two groups. The control group consisted of 278 normozoospermic individuals, according to the inclusion criteria of *World Health Organization* described in the Introduction (volume > 1.5 mL, sperm concentration > 39 million/mL, total sperm number > 15 million, total motility > 40%, progressive motility > 32% and viability > 58%). The case group consisted of 298 non-normozoospermic individuals. These were either oligozoospermic individuals, with a total sperm number of less than 15 million/mL, or asthenozoospermic individuals with motility less than 40%, or teratozoospermic individuals with less than 4% normal morphology, or combinations of the three categories.

It should also be noted that the samples were collected for the research program *"Spermogene"* that aims to determine the genetic profile of male infertility in each of its expressions, and then to develop a valuable diagnostic tool that can reliably assess male fertility with a simple blood sample from the male in question.

### *DNA Extraction*

DNA was extracted from semen samples according to the protocol developed by Weyrich, 2012. In brief, ethanol was used to remove seminal fluid from samples as it can affect the DNA quality, and after that, cell lysis followed. Lysis buffer contained 10 mM Tris-HCl pH 8.0, 100 mM NaCl, 10 mM EDTA, 0.5% SDS Triton-X100 0.5%, and DDT to break the disulfide bonds of the lipidic membrane that protects spermatozoa. It also contained proteinase K and SDS to ensure cell lysis and protein destruction. Centrifugation was used to remove the cell debris and then, the supernatant was carefully collected. To obtain DNA of high quality, an extra step was also performed. Thus, phenol-chloroform extraction was used to fully separate nucleic acid from proteins and lipids. The mixture of phenol-chloroform is immiscible with water and as a result, two phases are created. The hydrophilic lipids are partitioned into the lower organic phase, the proteins remain at the interphase and the nucleic acids are in the aqueous phase. Thus, the aqueous phase was collected, and the DNA was then precipitated with the use of ethanol, washed, and resuspended in dd$H_2O$.

DNA quality was assessed by agarose gel electrophoresis and quantity was assessed spectrophotometrically. It was subsequently adjusted to approximately 200 ng/mL.

As the samples were collected for the research program "Spermogene", DNA extractions of many samples were also performed by Maria Markantoni, Ph.D. candidate (Genomics: Exploring the causes of male infertility) of the Laboratory of Genetics, Comparative and Evolutionary Biology, UTH.

### *Genotyping*

All the DNA samples were genotyped at the Human Genomics Facility (HuGe-F) of Erasmus MC (University Medical Centre Rotterdam, Netherlands) using the Illumina Infinium® Global Screening Array which contains 756.388 single-nucleotide polymorphisms (SNPs) across the human genome.

### *Quality Control*

The genotype data that were obtained, in. ped and .map files, were analyzed using PLINK (https://zzz.bwh.harvard.edu/plink/) a software package with a wide range of functions, that is widely used for the analysis of GWAS data (Purcell et al., 2007).

Regarding quality control (QC), genotyping efficiency or call rate as it is called is an important issue. More specifically, when a large proportion of SNP assays fail on an individual DNA sample, this could be indicative of a DNA sample with poor quality. Thus, samples with low genotyping efficiency should be removed from further analysis. This can be done by using the - - *mind* option in PLINK. Marker genotyping efficiency is also used to assess the quality of the markers. SNP assays that fail on many samples are considered poor assays as they can affect the accuracy of the results. Thus, the - - *geno* option in PLINK is used to remove markers based on the call rate. Finally, it is also important to remove SNPs based on Minor Allele Frequency (MAF) as statistical power is extremely low for rare SNPs. The - - *maf* option in PLINK is usually recommended for this removal.

Therefore, all SNPs analyzed met the following quality control criteria: SNPs with MAF ≤0.05 were excluded, individuals with more than 10% missing genotypes were excluded and only SNPs with 90% genotyping rate were included in the study. The command used for quality control was:

*plink - - file [input_data] - - geno 0.1 - - mind 0.1 - - maf 0.05 - -tab - - recode - - out [data_produced]*

### *Association Analysis*

After the QC, files containing genotypes for thousands of SNPs in hundreds of samples are produced. The aim is to perform a statistical evaluation of the association of genotype with the phenotype of interest.

In this study, the two main groups were cases (non-normozoospermic) and controls (normozoospermic) but cases could also further subdivide into three more categories, oligozoospermic, asthenozoospermic, and teratozoospermic, as described in *Patient Selection and Biological Material*. Thus, for the SNP association analysis, Pearson's chi-square test was used to compare allele frequencies between four different comparisons (Normozoospermic vs Non-Normozoospermic, Normozoospermic vs Oligozoospermic, Normozoospermic vs Oligozoospermic, and Normozoospermic vs Teratozoospermic) and to test the null hypothesis that there is no association between each SNP and the phenotype of interest. For the evaluation of SNPs, the threshold of *p-value* ≤ 0.05 was used. In a GWAS, *p-value* provides statistical significance and is used as a safeguard against false positives. Odds ratio (OR) was also used in combination with *p-values* to evaluate the strength and the type of association. More specifically, OR = 1 implies no association between phenotype and genotype, OR < 1 implies that the allele is protective against the disease, and OR > 1 implies that the allele is associated with increased risk for the disease, meaning male infertility.

The command used for the four association analyses was: *plink - - file [file_name] - - assoc*

### *Bioinformatics Analyses*

This study aimed at identifying SNPs on Glycolysis and OXPHOS genes associated with male infertility or specific subtypes of infertility (oligozoospermic, asthenozoospermic, teratozoospermic). Thus, the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html) was used to identify all the genes of Glycolysis Pathway (*Table 2*) and the HUGO Gene Nomenclature Committee (HGNC) database (https://www.genenames.org/data/genegroup/#!/group/639) was also used to identify all OXPHOS genes, nuclear and mt-encoded (*Table 3*).

*Table 2: Genes of Glycolysis pathway according to KEGG Pathway database*

| Glycolysis Pathway Genes | | | | | |
|---|---|---|---|---|---|
| *ACSS1* | *ALDH1B1* | *DLAT* | *GCK* | *PCK1* | *PGAM1* |
| *ACSS2* | *ALDH2* | *DLD* | *GPI* | *PCK2* | *PGAM2* |
| *ADH1A* | *ALDH3A1* | *ENO1* | *HK1* | *PDHA1* | *PGAM4* |
| *ADH1B* | *ALDH3A2* | *ENO2* | *HK2* | *PDHA2* | *PGK1* |
| *ADH1C* | *ALDH3B1* | *ENO3* | *HK3* | *PDHB* | *PGK2* |
| *ADH4* | *ALDH3B2* | *FBP1* | *HKDC1* | *PFKFB1* | *PGM1* |
| *ADH5* | *ALDH7A1* | *FBP2* | *LDHA* | *PFKFB2* | *PGM2* |
| *ADH6* | *ALDH9A1* | *G6PC* | *LDHAL6A* | *PFKFB3* | *PKLR* |
| *ADH7* | *ALDOA* | *G6PC2* | *LDHAL6B* | *PFKFB4* | *PKM* |
| *ADPGK* | *ALDOB* | *GALM* | *LDHB* | *PFKL* | *SLC2A2* |
| *AKR1A1* | *ALDOC* | *GAPDH* | *LDHC* | *PFKM* | *TPI1* |
| *ALDH1A3* | *BPGM* | *GAPDHS* | *PANK1* | *PFKP* | |

*Table 3: Genes of Mitochondrial Respiratory Complexes (OXPHOS), as found using the HGNC database*

| Mitochondrial Respiratory Chain Complexes/OXPHOS genes | | | | | |
|---|---|---|---|---|---|
| **Mt. Complex I** | | **Mt. Complex II** | **Mt. Complex III** | **Mt. Complex IV** | **Mt. Complex V** |
| *MT-ND1* | *NDUFAB1* | *SDHA* | *UQCRB* | *COX4I1* | *ATP5F1A* |
| *MT-ND2* | *NDUFA1* | *SDHB* | *UQCRQ* | *COX4I2* | *ATP5F1B* |
| *MT-ND3* | *NDUFA2* | *SDHC* | *UQCRC1* | *COX5A* | *ATP5F1C* |
| *MT-ND4* | *NDUFA3* | *SDHD* | *UQCRC2* | *COX5B* | *ATP5F1D* |
| *MT-ND4L* | *NDUFA5* | | *MT-CYB* | *COX6A1* | *ATP5F1E* |
| *MT-ND5* | *NDUFA6* | | *CYC1* | *COX6A2* | *ATP5MC1* |
| *MT-ND6* | *NDUFA7* | | *UQCRFS1* | *COX6B1* | *ATP5MC2* |
| *NDUFS1* | *NDUFA8* | | *UQCRH* | *COX6B2* | *ATP5MC3* |
| *NDUFS2* | *NDUFA9* | | *UQCR10* | *COX6C* | *ATP5ME* |
| *NDUFS3* | *NDUFA10* | | *UQCR11* | *COX7A1* | *ATP5MF* |
| *NDUFS7* | *NDUFA11* | | | *COX7A2* | *ATP5MG* |

| | | | | | |
|---|---|---|---|---|---|
| *NDUFS8* | *NDUFA12* | | | *COX7B* | *ATP5MJ* |
| *NDUFV1* | *NDUFA13* | | | *COX7B2* | *ATP5MK* |
| *NDUFV2* | *NDUFB3* | | | *COX7C* | *MT-ATP6* |
| *NDUFC1* | *NDUFB4* | | | *COX8A* | *MT-ATP8* |
| *NDUFC2* | *NDUFB5* | | | *COX8C* | *ATP5PB* |
| *NDUFS4* | *NDUFB6* | | | *MT-CO1* | *ATP5PD* |
| *NDUFS5* | *NDUFB7* | | | *MT-CO2* | *ATP5PF* |
| *NDUFS6* | *NDUFB8* | | | *MT-CO3* | *ATP5PO* |
| *NDUFV3* | *NDUFB9* | | | | *ATP5IF1* |
| *NDUFB1* | *NDUFB10* | | | | |
| *NDUFB2* | *NDUFB11* | | | | |

After that, PLINK was used to identify all SNPs found on these genes and according to the results of the association analyses for the four comparisons described above, SNPs on Glycolysis and OXPHOS genes associated with male infertility or specific subtypes of infertility (oligozoospermia, asthenozoospermia, teratozoospermia) were identified. After that, to assess the potential role of these SNPs to male infertility, the Ensembl GRCh37 database (http://grch37.ensembl.org/index.html) and SNPnexus database (https://www.snp-nexus.org/v4/) were used to obtain information for each SNP regarding association with other diseases, and gene consequences (position and effect of variation on the specific gene).

The steps of the procedure that was followed during GWAS Analysis are presented in *Figure 14*.

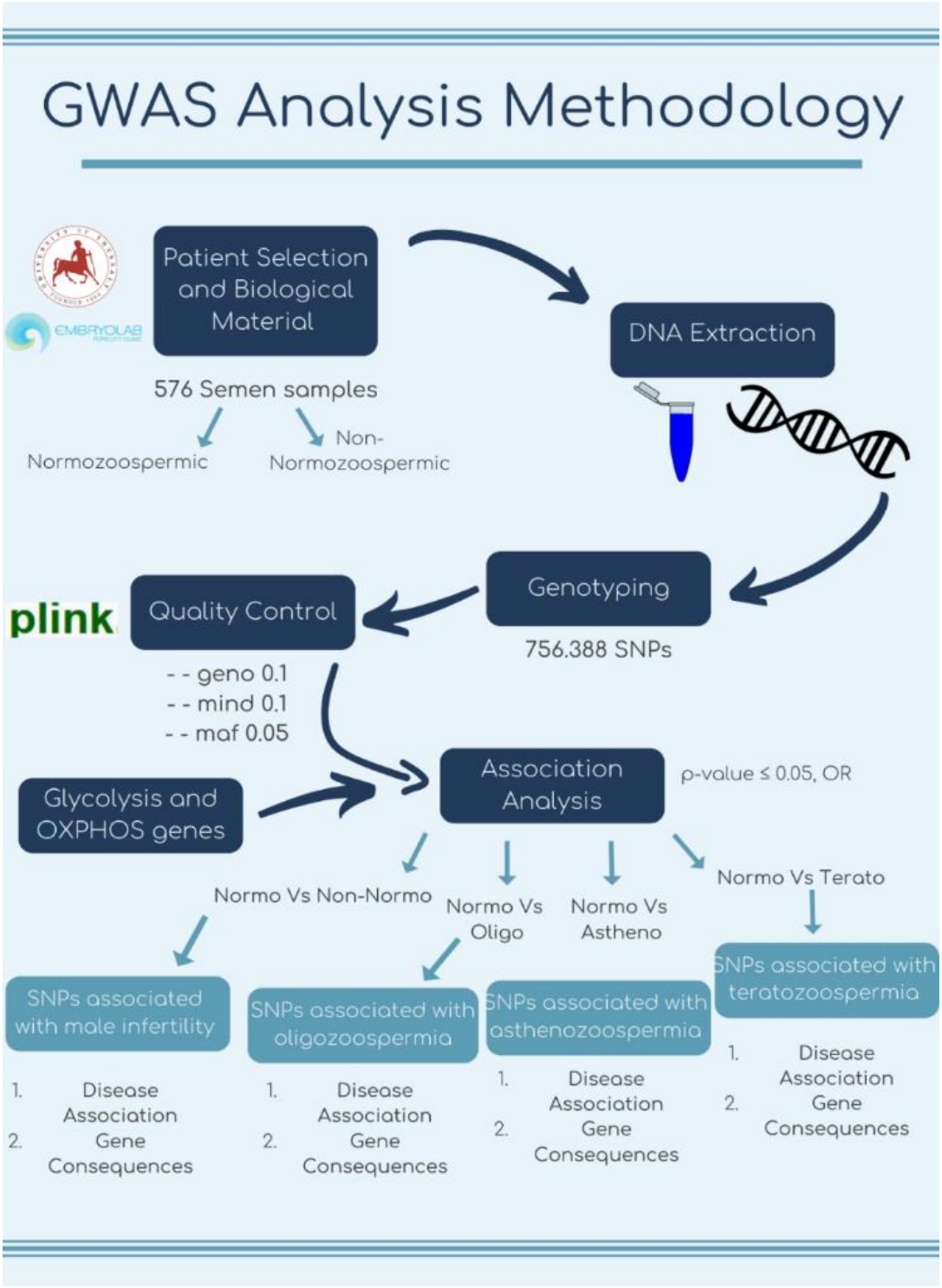


*Figure 14: Overview of the procedure that was followed during GWAS for identification of SNPs in glycolysis and OXPHOS genes associated with male infertility or with specific subtypes (asthenozoospermia, oligozoospermia, teratozoospermia).*

## B. WHOLE GENOME SEQUENCING (WGS) ANALYSIS

In Part B, Whole Genome Sequencing (WGS) Analysis of pooled samples characterized as normozoospermic or non-normozoospermic (asthenozoospermic, oligozoospermic, teratozoospermic) was used to identify variants in Glycolysis and OXPHOS genes.

Next-Generation Sequencing (NGS) has revolutionized the biological sciences as it enables the identification of novel or rare genetic factors that contribute to the phenotype of interest (pathologic conditions, diseases, etc.). The introduction of next-generation sequencing instrumentation has enhanced the capacity to perform whole-genome sequencing (WGS) in a rapid and cost-efficient manner (Krausz & Riera-Escamilla, 2018). In general, WGS is a process that determines the entire DNA sequence of a person's genome at once and it involves several steps as presented in *Figure 15*. WGS can be used for the identification of genetic variation including single nucleotide polymorphisms (SNPs), indels (insertions or deletions), copy number variations (CNVs), etc.

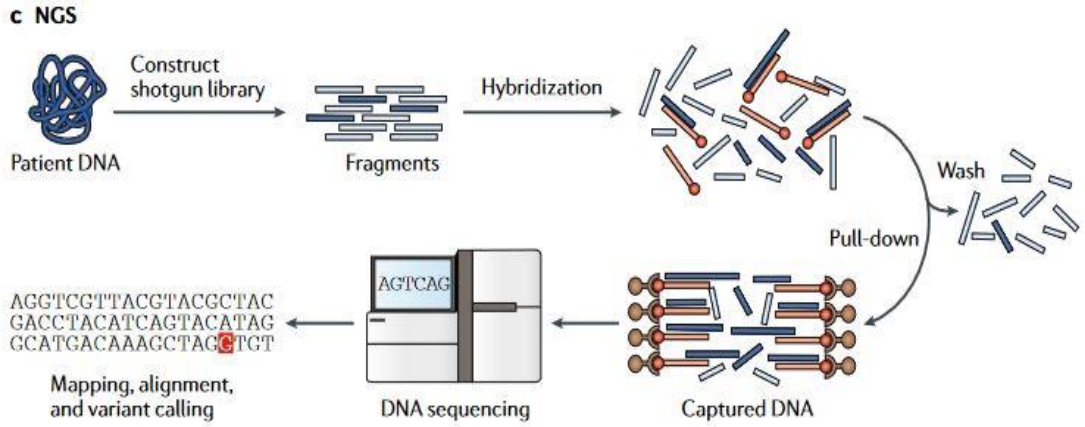


*Figure 15: Steps required during a typical Whole Genome Sequencing Analysis* (Krausz & Riera-Escamilla, 2018).

### *Data Preparation and Sequencing*

As in Part A, semen, as well as blood samples, were collected in cooperation with the "Embryolab Fertility Clinic" (Thessaloniki, Greece) from Greek individuals, aged between 18-40 years, who have given approved informed consent. Semen analysis was performed for all samples to obtain information about the phenotype. Thus, samples were divided into categories according to the results of the semen analysis. Normozoospermic individuals have defined according to the inclusion criteria of the *World Health Organization* described in the Introduction (volume > 1.5 mL, sperm concentration > 39 million/mL, total sperm number > 15 million, total motility > 40%, progressive motility > 32% and viability > 58%) and non-normozoospermic individuals were either oligozoospermic individuals, with a total sperm number of less than 15 million/mL, or asthenozoospermic individuals with motility less than 40%, or teratozoospermic individuals with less than 4% normal morphology, or combinations of the three categories.

Blood samples were collected, and DNA extraction was performed. For DNA extraction, PureLink Genomic DNA Mini Kit (Invitrogen) was used according to manufacturers' protocol. DNA quality and quantity were assessed by agarose gel electrophoresis and in Qubit using Qubit dsDNA BR Assay Kit (Invitrogen), respectively. After that, four sequencing pools were created. The first contained pooled DNAs from 10 normozoospermic individuals and the second contained pooled DNAs from 5 oligozoospermic individuals. Finally, the third and fourth contained pooled DNAs from 5 asthenozoospermic and 5 teratozoospermic individuals, respectively. Then, the DNA samples were shipped to Novogene where 100-bp paired-end libraries were constructed and they were sequenced using an Illumina HiSeq 3000 in a mean sequencing coverage of 30x. Library preparation is also a procedure consisting of several steps including fragmentation of the genomic material, ligation of oligonucleotide adapters to the ends of the fragments, enrichment, etc. The output of sequencing was a FASTQC file. The quality of the reads was assessed using FASTQC (Andrews, 2010) and after that, sequences with low quality (minimum PHRED Score: 30 ) were discarded using Trimmomatic (Bolger et al., 2014).

All these samples were also collected for the research program "Spermogene", as described in Part A of the analysis.

### *Alignment and assembly*

For the alignment, the reference genome was retrieved from the Ensembl database (http://ftp.ensembl.org/pub/grch37/grch37/current/fasta/homo_sapiens/dna/) and more specifically, Human Genome GRCh37 was selected as the same genome was used also for variant identification during GWAS (Part A). Thus, the reads of the samples were mapped to the reference genome. Whole-genome assembly followed the step of sequence alignment for the construction of a consensus sequence.

### *Variant Calling*

Variant calling is one of the most important steps in the WGS pipeline as it is used to identify differences between the sequence of interest and reference sequence at a given position in an individual genome. Therefore, after genome alignment and assembly, variant calling was performed using SAMtools/BCFtools (Li et al., 2009) and as an output, the results were stored in a standardized format called variant call format (vcf).

### *Annotation and Analysis*

Variant annotation is another crucial step used to retrieve biological information as the variants identified in the previous steps are matched to gene sequences from existing databases. For the annotation, the VEP tool provided by Ensembl (McLaren et al., 2016) was used (http://grch37.ensembl.org/Tools/VEP) and information about their location and consequence, as well as association with the phenotype of interest, meaning male infertility, was obtained.

The pipeline used for WGS analysis is presented in *Figure 16*.

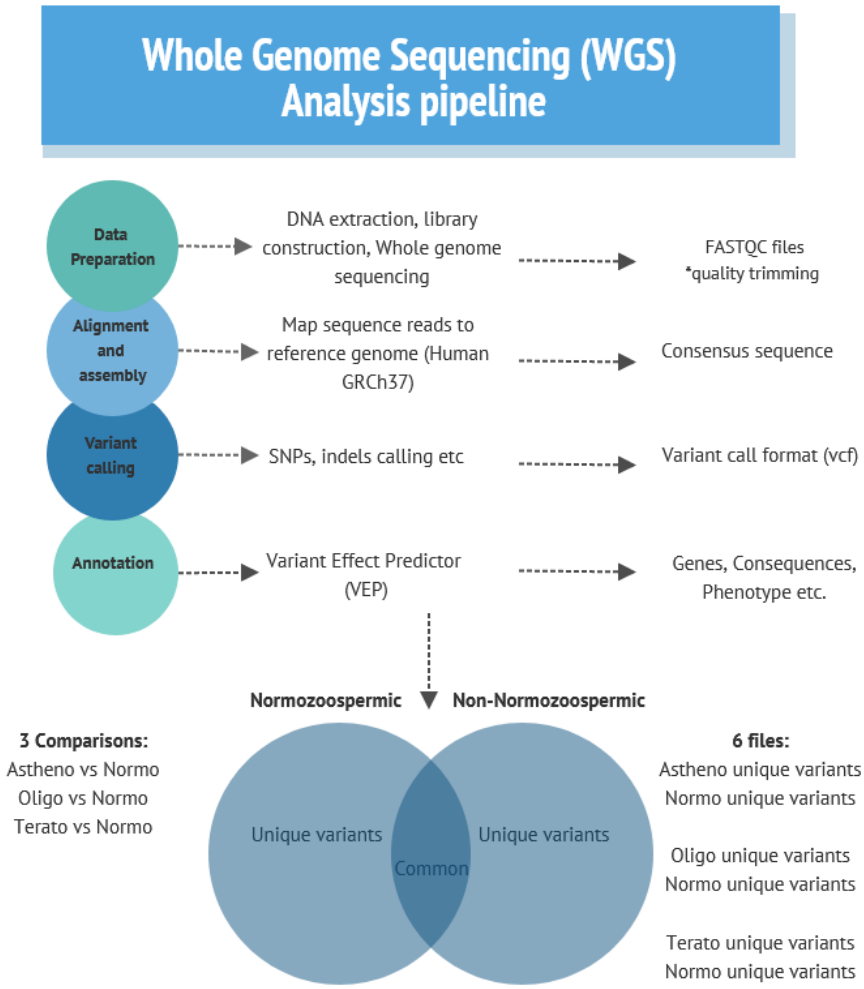


*Figure 16: Whole Genome Sequencing Analysis pipeline used to study variants in normozoospermic and non-normozoospermic individuals.*

Furthermore, BCFtools was used to compare the vcf files from normozoospermic and non-normozoospermic individuals in order to detect variants that are present only in one of the two groups, thus, they are not shared between normozoospermic and non-normozoospermic. More specifically, three comparisons were performed: asthenozoospermic vs normozoospermic, oligozoospermic vs normozoospermic, and teratozoospermic vs normozoospermic.

A custom script was also written to identify from all the variants identified above, only those that are found on Glycolysis and OXPHOS genes. This was performed for every comparison described. Finally, another script was written to detect unique genes in each pool of SNPs (i.e., genes with mutations only in one of the two groups, normozoospermic or non-normozoospermic, etc.).

These steps required for the identification of variants on glycolysis and OXPHOS genes are presented in *Figure 17*.

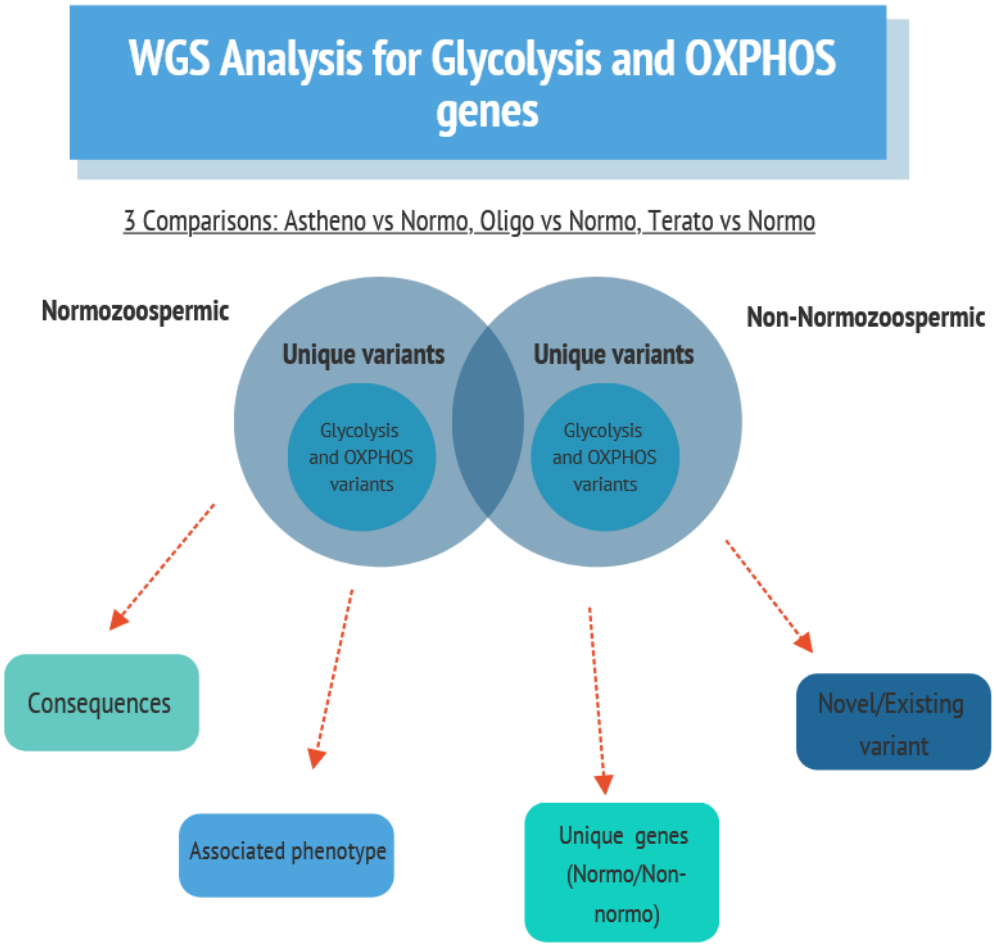


*Figure 17: WGS Analysis for the identification of SNPs in Glycolysis or OXPHOS genes between normozoospermic and non-normozoospermic individuals.*

# Results

## A. GENOME-WIDE ASSOCIATION STUDY (GWAS)

The association between Glycolysis or OXPHOS genes and male infertility or different subtypes of male infertility (asthenozoospermia, oligozoospermia, teratozoospermia) is still largely unexplored. Thus, GWAS was used to identify and list SNPs significantly associated with male infertility, asthenozoospermia, oligozoospermia, or teratozoospermia, by performing four comparisons as described above (Normozoospermic vs Non-Normozoospermic, Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic). Of all the SNPs found as statistically significant, SNPs on Glycolysis and OXPHOS genes were selected for further study. Thus, at first, results regarding Glycolysis genes are presented and results for OXPHOs genes follow.

### *Association of SNPs in Glycolysis genes with male infertility (Normozoospemic vs Non-Normozoospermic)*

For the first comparison, 278 normozoospermic (control group) and 298 non-normozoospermic (case group) individuals were selected for genotyping using the Illumina Infinium® Global Screening Array. Genotyping revealed variance among 756.388 single-nucleotide polymorphisms (SNPs) across the human genome. Meanwhile, it was observed that some SNPs failed to be scored on at least 90% of all the individuals and had a MAF ≤ 0.05 in the whole dataset. Some individuals also had more than 10% missing genotypes. By excluding the SNPs and individuals described above, after Quality Control, the remaining 299.992 SNPs and 488 individuals (control group, n = 248; case group, n = 240) were used for the GWAS analysis. The case group consisted of oligozoospermic, asthenozoospermic, teratozoospermic, and their combinations individuals.

After association analysis was performed, of approximately 340 SNPs found on genes of the Glycolysis Pathway, 17 SNPs were found as significantly associated with male infertility because they satisfied the criteria for genome-wide significance (*p-value* ≤ 0.05 and odds ratio). Ensembl GRCh37 (Howe et al., 2021) and SNPnexus databases (Oscanoa et al., 2020) were also used to obtain information for each SNP regarding association with other diseases, and gene consequences (position and effect of variation on the specific gene). The results are presented in *Table 4*.

*Table 4: SNPs associated with male infertility, p-value, odds ratio, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Gene | Consequence | Associated Diseases |
|---|---|---|---|---|---|---|---|
| 10 | rs57770060 | 9.581 | 0.001966 | 1.496 | *PFKB3* | intronic, NMD transcript variant | No |
| 10 | rs61173401 | 7.926 | 0.004873 | 0.6059 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 10 | rs1227938 | 6.795 | 0.009142 | 0.7017 | *HK1* | intronic, non-coding | Acquired Immunodeficiency Syndrome |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | transcript variant | |
| 6 | rs696718 | 6.723 | 0.009519 | 0.6893 | *PGK2* | 3 downstream | Acquired Immunodeficiency Syndrome |
| 4 | rs1154469 | 6.378 | 0.01155 | 0.7063 | *ADH7* | splice region variant, intronic | No |
| 2 | rs681900 | 6.353 | 0.01172 | 1.431 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 2 | rs3821310 | 5.916 | 0.015 | 0.5675 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 1 | rs3748671 | 5.84 | 0.01566 | 0.7324 | *PFKFB2* | 3 prime utr | No |
| 1 | rs74927590 | 5.716 | 0.01682 | 0.6408 | *PGM1* | intronic | No |
| 4 | rs2911907 | 5.213 | 0.02242 | 1.426 | *PGM2* | intronic, NMD transcript variant | No |
| 4 | rs2608307 | 5.174 | 0.02292 | 1.408 | *PGM2* | intronic, NMD transcript variant | No |
| 2 | rs11893422 | 4.792 | 0.02859 | 1.343 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 4 | rs2714559 | 4.684 | 0.03045 | 1.385 | *PGM2* | intronic, NMD transcript variant | No |
| 11 | rs1406580 | 4.526 | 0.03337 | 1.488 | *LDHC* | intronic, NMD transcript variant | No |
| 10 | rs7896691 | 4.363 | 0.03673 | 1.739 | *PFKP* | intronic | No |
| 10 | rs76124111 | 4.243 | 0.03941 | 0.5974 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 19 | rs17705657 | 3.991 | 0.04575 | 1.345 | *GAPDHS* | intronic | No |

### *Association of SNPs in Glycolysis genes with asthenozoospermia (Normozoospermic vs Asthenozoospermic)*

For the second comparison, 270 normozoospermic (control group) and 5 asthenozoospermic (case group) individuals were selected for genotyping which revealed variance among 756.388 SNPs across the human genome. After Quality Control, 299.600 SNPs and 242 individuals (control group, n = 237; case group, n = 5) remained for the GWAS analysis.

According to the results of the association analysis, of approximately 340 SNPs found on genes of the Glycolysis Pathway, 11 SNPs were found as significantly associated with asthenozoospermia, and as in previous comparisons, information for each SNP and results are presented in *Table 5*.

*Table 5: SNPs associated with asthenozoospermia, p-value, odds ratio, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Gene | Consequence | Associated Diseases |
|---|---|---|---|---|---|---|---|
| 10 | rs2305198 | 7.692 | 0.005548 | 5.624 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 11 | rs115420416 | 7.039 | 0.007974 | 5.445 | *LDHAL6A* | intronic | No |
| 10 | rs7076408 | 6.668 | 0.009814 | 5.089 | *PFKFB3* | intronic, non-coding transcript variant | No |
| 10 | rs1227938 | 6.154 | 0.01311 | 0 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 10 | rs7072268 | 5.783 | 0.01619 | 5.528 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 10 | rs1867974 | 5.524 | 0.01876 | 0.123 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 12 | rs2238151 | 5.372 | 0.02047 | 4.411 | *ALDH2* | intronic, NMD transcript variant | Acquired Immunodeficiency Syndrome |
| 10 | rs10740318 | 4.876 | 0.02724 | 3.768 | *HK1* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 3 | rs9810991 | 4.269 | 0.03882 | 4.667 | *PFKFB4* | intronic, non-coding transcript variant, NMD transcript variant | Menopause |
| 2 | rs11893422 | 4.239 | 0.03952 | 3.543 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 10 | rs2182409 | 3.99 | 0.04576 | 0 | *PFKFB3* | intronic | No |

### *Association of SNPs in Glycolysis genes with oligozoospermia (Normozoospermic vs Oligozoospermic)*

Furthermore, 270 normozoospermic (control group) and 57 oligozoospermic (case group) individuals were genotyped for 756.388 SNPs across the human genome. Quality Control was performed to exclude SNPs and individuals as described above, and the remaining 301.275 SNPs and 289 individuals (control group, n = 237; case group, n = 52) were used for the GWAS analysis.

After association analysis was performed, of approximately 340 SNPs found on genes of the Glycolysis Pathway, 16 SNPs were found as significantly associated with oligozoospermia (*p-value* ≤ 0.05 and odds ratio). The SNPs and information about their association with other diseases and gene consequences (position and effect of variation on the specific gene) are presented in *Table 6*.

*Table 6: SNPs associated with oligozoospermia, p-value, odds ratio, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Gene | Consequence | Associated Diseases |
|---|---|---|---|---|---|---|---|
| 2 | rs681900 | 6.604 | 0.01017 | 1.797 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 10 | rs10881606 | 5.775 | 0.01625 | 1.697 | *PANK1* | intronic, non-coding transcript variant | No |
| 17 | rs76846683 | 5.625 | 0.01771 | 2.276 | *ALDH3A2* | 3 prime UTR variant, NMD transcript variant, non-coding transcript exon variant | Acquired Immunodeficiency Syndrome |
| 5 | rs2278492 | 5.521 | 0.01879 | 0.5644 | *HK3* | synonymous variant, non-coding transcript exon variant, intronic, NMD transcript variant | Age at menarche/menopause, Acquired Immunodeficiency syndrome |
| 4 | rs6844466 | 5.509 | 0.01892 | 1.927 | *PGM2* | intronic, NMD transcript variant | No |
| 10 | rs11251717 | 5.133 | 0.02348 | 0.5792 | *PFKP* | intronic, non-coding transcript variant | No |
| 9 | rs533017 | 5.093 | 0.02402 | 1.662 | *ALDOB* | intronic | No |
| 10 | rs10903966 | 4.586 | 0.03224 | 1.761 | *PFKP* | intronic, non-coding transcript variant | No |
| 15 | rs4646649 | 4.476 | 0.03438 | 1.783 | *ALDH1A3* | intronic, non-coding transcript variant, NMD transcript variant | No |
| 10 | rs7899214 | 4.467 | 0.03456 | 0.2386 | *HKDC1* | intronic | No |
| 12 | rs1060619 | 4.241 | 0.03947 | 0.4362 | *GAPDH* | intronic, non-coding transcript variant | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | rs2038921 | 4.109 | 0.04266 | 1.557 | *PANK1* | intronic | No |
| 9 | rs10993269 | 4.026 | 0.04481 | 0.6333 | *FBP1* | intronic | No |
| 1 | rs1143661 | 3.89 | 0.04859 | 2.018 | *ALDH9A1* | synonymous variant, non-coding transcript exon variant | Acquired Immunodeficiency Syndrome |
| 10 | rs4750017 | 3.865 | 0.04931 | 1.88 | *PFKFB3* | intronic | No |
| 10 | rs618982 | 3.834 | 0.05021 | 0.5676 | *PFKFB3* | intronic | No |

### *Association of SNPs in Glycolysis genes with teratozoospermia (Normozoospermic vs Teratozoospermic)*

For the last comparison, 270 normozoospermic (control group) and 51 teratozoospermic (case group) individuals were genotyped for 756.388 SNPs across the human genome and after the Quality Control, as described above, the remaining 300.709 SNPs and 286 individuals (control group, n = 237; case group, n = 49) were used for the GWAS analysis.

After association analysis was performed, of approximately 340 SNPs found on genes of the Glycolysis Pathway, 21 SNPs were found as significantly associated with teratozoospermia. The results are presented in *Table 7*.

*Table 7: SNPs associated with teratozoospermia, p-value, odds ratio, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Gene | Consequence | Associated Diseases |
|---|---|---|---|---|---|---|---|
| 17 | rs2593595 | 11.2 | 0.0008183 | 2.251 | *G6PC* | intronic, non-coding transcript exon variant | SIDS/sudden infant death syndrome |
| 4 | rs2714559 | 8.07 | 0.0045 | 1.962 | *PGM2* | intronic, NMD transcript variant | No |
| 4 | rs2608307 | 7.937 | 0.004845 | 1.951 | *PGM2* | intronic, NMD transcript variant | No |
| 11 | rs1830426 | 7.432 | 0.006409 | 1.846 | *LDHA* | intronic, NMD transcript variant, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 11 | rs1406580 | 7.289 | 0.006938 | 2.133 | *LDHC* | intronic, NMD transcript variant | No |
| 2 | rs11893422 | 6.415 | 0.01131 | 1.771 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 4 | rs2911907 | 6.074 | 0.01372 | 1.832 | *PGM2* | intronic, NMD transcript variant | No |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | rs10795005 | 5.967 | 0.01457 | 0.3788 | *PFKP* | intronic, non-coding transcript variant | No |
| 10 | rs2279208 | 5.759 | 0.0164 | 2.044 | *PFKP* | intronic | No |
| 9 | rs7859877 | 5.637 | 0.01759 | 1.713 | *ALDH1B1* | 3 prime utr variant | Acquired Immunodeficiency Syndrome - Varicose Veins |
| 9 | rs2073478 | 5.326 | 0.02101 | 1.677 | *ALDH1B1* | missense variant | Acquired Immunodeficiency Syndrome |
| 10 | rs17151529 | 5.299 | 0.02133 | 0.4871 | *PFKFB3* | intronic | No |
| 2 | rs13014787 | 4.775 | 0.02888 | 0.4538 | *GALM* | intronic, NMD transcript variant | No |
| 10 | rs4881077 | 4.62 | 0.0316 | 0.5183 | *PFKP* | intronic, non-coding transcript variant | No |
| 10 | rs11592061 | 4.472 | 0.03446 | 0.578 | *PFKP* | intronic, non-coding transcript variant | No |
| 2 | rs634849 | 4.028 | 0.04476 | 1.626 | *HK2* | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 21 | rs8133730 | 4.025 | 0.04482 | 1.698 | *PFKL* | intronic, NMD transcript variant, non-coding transcript variant | No |
| 2 | rs10496195 | 3.995 | 0.04565 | 0.6114 | *HK2* | intronic | Acquired Immunodeficiency Syndrome |
| 1 | rs2269237 | 3.973 | 0.04625 | 0.3609 | *PGM1* | intronic, non-coding transcript variant | No |
| 5 | rs34241005 | 3.839 | 0.05008 | 1.653 | *ALDH7A1* | intronic, NMD transcript variant, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 3 | rs13069849 | 3.832 | 0.05028 | 0.2613 | *SLC2A2* | intronic, NMD transcript variant | No |

### *Genome-Wide Association Analysis and Glycolysis Genes*

In total, all the SNPs found on Glycolysis genes, identified as statistically significant for the comparisons that were performed, are presented in *Table 8*. As it is observed, some of them were found as statistically significant in more than one category but most of them were found as statistically significant only in one comparison.

*Table 8: SNPS on Glycolysis genes identified as statistically significant and associated with male infertility, or its subcategories, asthenozoospermia, oligozoospermia, or teratozoospermia. For every comparison and every SNP, p-value (P) and odds ratio (OR) are presented. Some SNPs were identified as statistically significant in more than one comparison.*

| | | Normozoospermic Vs Non-Normozoospermic | | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|---|---|---|
| **SNPs** | **Gene** | **P** | **OR** | **P** | **OR** | **P** | **OR** | **P** | **OR** |
| **rs57770060** | *PFKB3* | 0.001966 | 1.496 | | | | | | |
| **rs61173401** | *HK1* | 0.004873 | 0.6059 | | | | | | |
| **rs1227938** | *HK1* | 0.009142 | 0.7017 | 0.01311 | 0 | | | | |
| **rs696718** | *PGK2* | 0.009519 | 0.6893 | | | | | | |
| **rs1154469** | *ADH7* | 0.01155 | 0.7063 | | | | | | |
| **rs681900** | *HK2* | 0.01172 | 1.431 | | | 0.01017 | 1.797 | | |
| **rs3821310** | *HK2* | 0.015 | 0.5675 | | | | | | |
| **rs3748671** | *PFKFB2* | 0.01566 | 0.7324 | | | | | | |
| **rs74927590** | *PGM1* | 0.01682 | 0.6408 | | | | | | |
| **rs2911907** | *PGM2* | 0.02242 | 1.426 | | | | | 0.01372 | 1.832 |
| **rs2608307** | *PGM2* | 0.02292 | 1.408 | | | | | 0.004845 | 1.951 |
| **rs11893422** | *HK2* | 0.02859 | 1.343 | 0.03952 | 3.543 | | | 0.01131 | 1.771 |
| **rs2714559** | *PGM2* | 0.03045 | 1.385 | | | | | 0.0045 | 1.962 |
| **rs1406580** | *LDHC* | 0.03337 | 1.488 | | | | | 0.006938 | 2.133 |
| **rs7896691** | *PFKP* | 0.03673 | 1.739 | | | | | | |
| **rs76124111** | *HK1* | 0.03941 | 0.5974 | | | | | | |
| **rs17705657** | *GAPDHS* | 0.04575 | 1.345 | | | | | | |
| **rs2305198** | *HK1* | | | 0.005548 | 5.624 | | | | |
| **rs115420416** | *LDHAL6A* | | | 0.007974 | 5.445 | | | | |
| **rs7076408** | *PFKFB3* | | | 0.009814 | 5.089 | | | | |
| **rs7072268** | *HK1* | | | 0.01619 | 5.528 | | | | |
| **rs1867974** | *HK1* | | | 0.01876 | 0.123 | | | | |
| **rs2238151** | *ALDH2* | | | 0.02047 | 4.411 | | | | |
| **rs10740318** | *HK1* | | | 0.02724 | 3.768 | | | | |
| **rs9810991** | *PFKFB4* | | | 0.03882 | 4.667 | | | | |
| **rs2182409** | *PFKFB3* | | | 0.04576 | 0 | | | | |
| **rs10881606** | *PANK1* | | | | | 0.01625 | 1.697 | | |
| **rs76846683** | *ALDH3A2* | | | | | 0.01771 | 2.276 | | |
| **rs2278492** | *HK3* | | | | | 0.01879 | 0.5644 | | |
| **rs6844466** | *PGM2* | | | | | 0.01892 | 1.927 | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **rs11251717** | *PFKP* | | | | | 0.02348 | 0.5792 | | |
| **rs533017** | *ALDOB* | | | | | 0.02402 | 1.662 | | |
| **rs10903966** | *PFKP* | | | | | 0.03224 | 1.761 | | |
| **rs4646649** | *ALDH1A3* | | | | | 0.03438 | 1.783 | | |
| **rs7899214** | *HKDC1* | | | | | 0.03456 | 0.2386 | | |
| **rs1060619** | *GAPDH* | | | | | 0.03947 | 0.4362 | | |
| **rs2038921** | *PANK1* | | | | | 0.04266 | 1.557 | | |
| **rs10993269** | *FBP1* | | | | | 0.04481 | 0.6333 | | |
| **rs1143661** | *ALDH9A1* | | | | | 0.04859 | 2.018 | | |
| **rs4750017** | *PFKFB3* | | | | | 0.04931 | 1.88 | | |
| **rs618982** | *PFKFB3* | | | | | 0.05021 | 0.5676 | | |
| **rs2593595** | *G6PC* | | | | | | | 0.0008183 | 2.251 |
| **rs1830426** | *LDHA* | | | | | | | 0.006409 | 1.846 |
| **rs10795005** | *PFKP* | | | | | | | 0.01457 | 0.3788 |
| **rs2279208** | *PFKP* | | | | | | | 0.0164 | 2.044 |
| **rs7859877** | *ALDH1B1* | | | | | | | 0.01759 | 1.713 |
| **rs2073478** | *ALDH1B1* | | | | | | | 0.02101 | 1.677 |
| **rs17151529** | *PFKFB3* | | | | | | | 0.02133 | 0.4871 |
| **rs13014787** | *GALM* | | | | | | | 0.02888 | 0.4538 |
| **rs4881077** | *PFKP* | | | | | | | 0.0316 | 0.5183 |
| **rs11592061** | *PFKP* | | | | | | | 0.03446 | 0.578 |
| **rs634849** | *HK2* | | | | | | | 0.04476 | 1.626 |
| **rs8133730** | *PFKL* | | | | | | | 0.04482 | 1.698 |
| **rs10496195** | *HK2* | | | | | | | 0.04565 | 0.6114 |
| **rs2269237** | *PGM1* | | | | | | | 0.04625 | 0.3609 |
| **rs34241005** | *ALDH7A1* | | | | | | | 0.05008 | 1.653 |
| **rs13069849** | *SLC2A2* | | | | | | | 0.05028 | 0.2613 |

### *Association of SNPs in OXPHOS genes with male infertility (Normozoospemic vs Non-Normozoospermic)*

GWAS Analysis was also performed to identify SNPs associated with male infertility or subtypes of male infertility located on OXPHOS genes, both nuclear and mt-encoded. As described in *Materials and Methods*, four comparisons were conducted.

For the first comparison, 278 normozoospermic (control group) and 298 non-normozoospermic (case group) individuals were genotyped for 756.388 SNPs and after Quality control, 299.992 SNPs and 488 individuals (control group, n = 248; case group, n = 240) were used for the GWAS analysis. The case group consisted of

oligozoospermic, asthenozoospermic, teratozoospermic, and their combinations individuals.

After association analysis was performed, of approximately 230 SNPs found on OXPHOS genes, 12 SNPs were found as significantly associated with male infertility (*p-value* ≤ 0.05 and odds ratio). Ensembl GRCh37 (Howe et al., 2021) and SNPnexus databases (Oscanoa et al., 2020) were also used to obtain information for each SNP regarding association with other diseases, and gene consequences (position and effect of variation on the specific gene). The results are presented in *Table 9*.

*Table 9: SNPs associated with male infertility, p-value, odds ratio (OR), mitochondrial complex, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Genes | Mt. Complex | Consequences | Associated Disease |
|---|---|---|---|---|---|---|---|---|
| 4 | rs13141126 | 13.24 | 0.0002745 | 1.753 | *COX7B2* | IV | intronic | Prostate Cancer |
| 8 | rs16899708 | 8.291 | 0.003983 | 2.283 | *NDUFB9* | I | intronic | Acquired Immunodeficiency Syndrome |
| 8 | rs4007921 | 7.633 | 0.00573 | 2.218 | *NDUFB9* | I | intronic | Acquired Immunodeficiency Syndrome |
| 10 | rs7911488 | 6.874 | 0.008745 | 0.7099 | *ATP5MD* | V | intronic, 5 utr | No |
| 12 | rs2240762 | 5.601 | 0.01795 | 1.36 | *NDUFA9* | I | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome, Prostate Cancer |
| 5 | rs702399 | 5.287 | 0.02148 | 0.7411 | *NDUFA2* | I | 3 downstream | Acquired Immunodeficiency Syndrome |
| 1 | rs11538340 | 4.704 | 0.0301 | 1.686 | *NDUFS2* | I | missense variant, non-coding transcript exon variant | Acquired Immunodeficiency Syndrome, Prostate Cancer |
| 18 | rs9955008 | 4.62 | 0.0316 | 1.318 | *ATP5F1A* | V | 5 upstream | No |
| 2 | rs13389248 | 4.385 | 0.03625 | 0.7246 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |
| 4 | rs1512126 | 4.283 | 0.0385 | 1.343 | *COX7B2* | IV | intronic | Prostate Cancer |
| 2 | rs7599067 | 4.152 | 0.04158 | 0.7077 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |
| 5 | rs3822362 | 4.08 | 0.0434 | 1.56 | *NDUFS6* | I | intronic | Immunodeficiency Syndrome, Prostate Cancer and Aging/Telomere Length |

### *Association of SNPs in OXPHOS genes with asthenozoospermia (Normozoospermic vs Asthenozoospermic)*

For the second comparison, 270 normozoospermic (control group) and 5 asthenozoospermic (case group) individuals were selected for genotyping which revealed variance among 756.388 SNPs across the human genome. After Quality Control, 299.600 SNPs and 242 individuals (control group, n = 237; case group, n = 5) remained for the GWAS analysis.

According to the results of the association analysis, of approximately 230 SNPs found on OXPHOS genes, 12 SNPs were found as significantly associated with asthenozoospermia, and as in previous comparisons, information for each SNP and results are presented in *Table 10*.

*Table 10: SNPs associated with asthenozoospermia, p-value, odds ratio (OR), mitochondrial complex, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Genes | Mt. Complex | Consequences | Associated Disease |
|---|---|---|---|---|---|---|---|---|
| 21 | rs4842 | 6.924 | 0.008506 | 5.376 | *ATP5O* | V | missense variant, intronic, NMD transcript variant, non-coding transcript exon variant | Acquired Immunodeficiency Syndrome, Prostate Cancer |
| 23 | rs4492499 | 6.15 | 0.01314 | NA | *NDUFB11* | I | intronic | No |
| 4 | rs1512126 | 5.709 | 0.01688 | 4.234 | *COX7B2* | IV | intronic | Prostate Cancer |
| 5 | rs3822362 | 5.402 | 0.02012 | 4.526 | *NDUFS6* | I | intronic | Acquired Immunodeficiency Syndrome, Prostate Cancer |
| 18 | rs2850644 | 4.794 | 0.02856 | 4.188 | *ATP5F1A* | V | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome, Prostate Cancer |
| 5 | rs7728496 | 4.613 | 0.03173 | 3.647 | *NDUFS4* | I | intronic, NDM transcript variant | Acquired Immunodeficiency Syndrome, Prostate Cancer, and Aging/Telomere Length |
| 19 | rs753420 | 4.55 | 0.03293 | 3.69 | *COX7A1* | IV | 5 prime UTR variant | Acquired Immunodeficiency Syndrome |
| 8 | rs3829038 | 4.433 | 0.03524 | 3.988 | *NDUFB9* | I | intronic, non-coding | Acquired Immunodeficiency Syndrome, |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | transcript variant | Prostate Cancer, and Aging/Telomere Length |
| 8 | rs77538674 | 4.433 | 0.03524 | 3.988 | *NDUFB9* | I | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome |
| 3 | rs35343040 | 4.298 | 0.03815 | 4.688 | *UQCRC1* | III | intronic, NDM transcript variant, non-coding transcript exon variant | Menopause, Acquired Immunodeficiency Syndrome |
| 11 | rs117711499 | 4.298 | 0.03815 | 4.688 | *SDHD* | II | intronic, NDM transcript variant | Acquired Immunodeficiency Syndrome |
| 16 | rs338791 | 3.916 | 0.04783 | 3.7 | *NDUFB10* | I | intronic, non-coding transcript variant | Acquired Immunodeficiency Syndrome and Aging/Telomere Length |

### *Association of SNPs in OXPHOS genes with oligozoospermia (Normozoospermic vs Oligozoospermic)*

Furthermore, 270 normozoospermic (control group) and 57 oligozoospermic (case group) individuals were genotyped for 756.388 SNPs and after Quality Control was performed to exclude SNPs and individuals as described above, the remaining 301.275 SNPs and 289 individuals (control group, n = 237; case group, n = 52) were used for the GWAS analysis.

After association analysis was performed, of approximately 230 SNPs found on OXPHOS genes, 10 SNPs were found as significantly associated with oligozoospermia (*p-value* ≤ 0.05 and odds ratio). The SNPs and information about their association with other diseases and gene consequences (position and effect of variation on the specific gene) are presented in *Table 11*.

*Table 11: SNPs associated with oligozoospermia, p-value, odds ratio (OR), mitochondrial complex, gene consequence and diseases associated with them*

| CHR | SNP | x square | p-value | OR | Genes | Mt. Complex | Consequences | Associated Disease |
|---|---|---|---|---|---|---|---|---|
| 12 | rs12818931 | 9.243 | 0.002364 | 0.1464 | *ATP5MC2* | V | 5 upstream | No |
| 10 | rs7911488 | 7.602 | 0.005829 | 0.5313 | *ATP5MD* | V | intronic, 5 utr | No |
| 12 | rs73218097 | 7.395 | 0.006539 | 1.806 | *NDUFA12* | I | intronic | No |
| 4 | rs13141126 | 7.338 | 0.006753 | 1.904 | *COX7B2* | IV | intronic | Prostate Cancer |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 19 | rs753420 | 5.843 | 0.01564 | 0.5149 | *COX7A1* | IV | 5 upstream | Acquired Immunodeficiency Syndrome |
| 1 | rs3768324 | 5.652 | 0.01744 | 1.708 | *NDUFS5* | I | intronic | Acquired Immunodeficiency Syndrome, Prostate Cancer, and Aging/Telomere Length |
| 4 | rs1512126 | 4.554 | 0.03285 | 1.625 | *COX7B2* | IV | intronic, 5 upstream | Prostate Cancer |
| 12 | rs2076022 | 4.273 | 0.03872 | 1.584 | *COX6A1* | IV | 5 upstream | No |
| 5 | rs72750143 | 4.236 | 0.03957 | 1.738 | *NDUFS4* | I | intronic | Acquired Immunodeficiency Syndrome Prostate Cancer and Aging/Telomere Length |
| 2 | rs77151027 | 3.862 | 0.04939 | 1.767 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |

### *Association of SNPs in OXPHOS genes with teratozoospermia (Normozoospermic vs Teratozoospermic)*

For the last comparison, 270 normozoospermic (control group) and 51 teratozoospermic (case group) individuals were genotyped for 756.388 SNPs across the human genome and after the Quality Control, as described above, the remaining 300.709 SNPs and 286 individuals (control group, n = 237; case group, n = 49) were used for the GWAS analysis.

After association analysis was performed, of approximately 230 SNPs found on OXPHOS genes, 7 SNPs were found as significantly associated with teratozoospermia. The results are presented in *Table 12*.

*Table 12: SNPs associated with teratozoospermia, p-value, odds ratio (OR), mitochondrial complex, gene consequence and diseases associated with them*

| CHR | SNP | X square | p-value | OR | Genes | Mt. Complex | Consequences | Associated Disease |
|---|---|---|---|---|---|---|---|---|
| 2 | rs4853981 | 10.08 | 0.001499 | 0.466 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |
| 2 | rs13402622 | 7.912 | 0.004911 | 0.4749 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 2 | rs11686460 | 5.094 | 0.02401 | 1.652 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |
| 2 | rs78826721 | 4.876 | 0.02723 | 0.3894 | *NDUFS1* | I | intronic | Acquired Immunodeficiency Syndrome, Prostate Cancer, and Aging/Telomere Length |
| 2 | rs7599067 | 4.696 | 0.03023 | 0.4718 | *NDUFA10* | I | intronic | Acquired Immunodeficiency Syndrome |
| 10 | rs7911488 | 4.658 | 0.03091 | 0.6069 | *ATP5MD* | V | intronic, 5 utr | No |
| 12 | rs249153 | 3.917 | 0.04781 | 0.5375 | *NDUFA12* | I | intronic | No |

### *Genome-Wide Association Analysis and OXPHOS Genes*

In total, all the SNPs found on OXPHOS genes and identified as statistically significant for the comparisons that were performed, are presented in *Table 13*. As it is observed, some of them were found as statistically significant in more than one category but most of them were found as statistically significant only in one comparison.

*Table 13: SNPS on OXPHOS genes identified as statistically significant and associated with male infertility, or its subcategories, asthenozoospermia, oligozoospermia, or teratozoospermia. For every comparison and every SNP, p-value (P) and odds ratio (OR) are presented. The mitochondrial complex is also represented next to every gene in parentheses. Some SNPs were identified as statistically significant in more than one comparison*

| | | Normozoospermic vs Non-Normozoospermic | | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|---|---|---|
| **SNPs** | **Gene** | **P** | **OR** | **P** | **OR** | **P** | **OR** | **P** | **OR** |
| **rs13141126** | *COX7B2 (IV)* | 0.0002745 | 1.753 | | | 0.006753 | 1.904 | | |
| **rs16899708** | *NDUFB9 (I)* | 0.003983 | 2.283 | | | | | | |
| **rs4007921** | *NDUFB9 (I)* | 0.00573 | 2.218 | | | | | | |
| **rs7911488** | *ATP5MD (V)* | 0.008745 | 0.7099 | | | 0.005829 | 0.5313 | 0.03091 | 0.6069 |
| **rs2240762** | *NDUFA9 (I)* | 0.01795 | 1.36 | | | | | | |
| **rs702399** | *NDUFA2 (I)* | 0.02148 | 0.7411 | | | | | | |
| **rs11538340** | *NDUFS2 (I)* | 0.0301 | 1.686 | | | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **rs9955008** | *ATP5F1A (V)* | 0.0316 | 1.318 | | | | | | |
| **rs13389248** | *NDUFA10 (I)* | 0.03625 | 0.7246 | | | | | | |
| **rs1512126** | *COX7B2 (IV)* | 0.0385 | 1.343 | 0.01688 | 4.234 | 0.03285 | 1.625 | | |
| **rs7599067** | *NDUFA10 (I)* | 0.04158 | 0.7077 | | | | | 0.03023 | 0.4718 |
| **rs3822362** | *NDUFS6 (I)* | 0.0434 | 1.56 | 0.02012 | 4.526 | | | | |
| **rs4842** | *ATP5O (V)* | | | 0.008506 | 5.376 | | | | |
| **rs4492499** | *NDUFB11 (I)* | | | 0.01314 | NA | | | | |
| **rs2850644** | *ATP5F1A (V)* | | | 0.02856 | 4.188 | | | | |
| **rs7728496** | *NDUFS4 (I)* | | | 0.03173 | 3.647 | | | | |
| **rs753420** | *COX7A1 (IV)* | | | 0.03293 | 3.69 | 0.01564 | 0.5149 | | |
| **rs3829038** | *NDUFB9 (I)* | | | 0.03524 | 3.988 | | | | |
| **rs77538674** | *NDUFB9 (I)* | | | 0.03524 | 3.988 | | | | |
| **rs35343040** | *UQCRC1 (III)* | | | 0.03815 | 4.688 | | | | |
| **rs117711499** | *SDHD (II)* | | | 0.03815 | 4.688 | | | | |
| **rs338791** | *NDUFB10 (I)* | | | 0.04783 | 3.7 | | | | |
| **rs12818931** | *ATP5MC2 (V)* | | | | | 0.002364 | 0.1464 | | |
| **rs73218097** | *NDUFA12 (I)* | | | | | 0.006539 | 1.806 | | |
| **rs3768324** | *NDUFS5 (I)* | | | | | 0.01744 | 1.708 | | |
| **rs2076022** | *COX6A1 (IV)* | | | | | 0.03872 | 1.584 | | |
| **rs72750143** | *NDUFS4 (I)* | | | | | 0.03957 | 1.738 | | |
| **rs77151027** | *NDUFA10 (I)* | | | | | 0.04939 | 1.767 | | |
| **rs4853981** | *NDUFA10 (I)* | | | | | | | 0.001499 | 0.466 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **rs13402622** | *NDUFA10 (I)* | | | | | | | 0.004911 | 0.4749 |
| **rs11686460** | *NDUFA10 (I)* | | | | | | | 0.02401 | 1.652 |
| **rs78826721** | *NDUFS1 (I)* | | | | | | | 0.02723 | 0.3894 |
| **rs7911488** | *ATP5MD (V)* | | | | | | | 0.03091 | 0.6069 |
| **rs249153** | *NDUFA12 (I)* | | | | | | | 0.04781 | 0.5375 |

## B. WHOLE GENOME SEQUENCING (WGS) ANALYSIS

As it has already been described, Whole Genome Sequencing Analysis was also used to identify and characterize variants in OXPHOS and Glycolysis Genes associated with male infertility and its subtypes by performing three comparisons (Asthenozoospermics vs Normozoospermics, Oligozoospermics vs Normozoospermics, and Teratozoospermics vs Normozoospermics).

As for the GWAS results, at first, results regarding the Glycolysis pathway are going to be presented and results for OXPHOS genes will follow.

### *Glycolysis Pathway*

At first, variants present only in one group (Cases or Control) for each comparison were identified (*Table 14*). Moreover, from the total SNPs found on every group, variants located only on Glycolysis genes were detected. Thus, the number of variants found on Glycolysis genes for every group studied and for every comparison is presented also in *Table 14*.

*Table 14: Total variants and variants found on Glycolysis genes for the three comparisons (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic)*

| | Asthenozoospermic vs Normozoospemic | | Oligozoospermic vs Normozoospemic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Total variants** | 1917404 | 6687079 | 2025316 | 6634959 | 1736484 | 6825542 |
| **Glycolysis variants** | 3294 | 8479 | 2745 | 9242 | 1716 | 9965 |
| **Glycolysis/Total variants (%)** | 0.172 | 0.127 | 0.136 | 0.139 | 0.001 | 0.001 |

Then, functional annotation of the unique variants for every group found on Glycolysis genes was also performed using the VEP tool to identify novel and existing variants. The results for every group and every comparison are presented in *Table 15*. Moreover, VEP was also used to study if any variant was associated according to previous research with male infertility or similar phenotype. None of the variants was associated with the phenotype of interest (*Table 15*).

*Table 15: Novel and existing variants for every group studied and every comparison. None of them was also found associated with the phenotype of interest according to VEP*

| | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Novel variants** | 196 | 621 | 226 | 818 | 205 | 1074 |
| **Existing variants** | 3098 | 7858 | 2519 | 8424 | 1511 | 8891 |
| **Phenotype of interest** | - | - | - | - | - | - |

The functional annotation of the unique variants found on Glycolysis genes and regarding their consequence (intronic, synonymous, missense variants, etc.) is also presented in *Table 16*. As it is observed, most of them are intronic variants.

*Table 16: Functional annotation of the unique variants found on Glycolysis genes for the three comparisons conducted (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic and Teratozoospermic vs Normozoospermic)*

| | Asthenozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| **Consequences** | **Astheno** | **Normo** | **Terato** | **Normo** | **Oligo** | **Normo** |
| **3 prime UTR var.** | 27 | 103 | 21 | 124 | 40 | 111 |
| **3 prime UTR var., NMD transcript var.** | 14 | 9 | 4 | 17 | 7 | 25 |
| **5 prime UTR var.** | 15 | 23 | 14 | 21 | 20 | 16 |
| **5 prime UTR var., NMD transcript var.** | 1 | 1 | - | 3 | 1 | 3 |
| **Intron var., NMD transcript var.** | 437 | 849 | 343 | 1176 | 374 | 1232 |
| **Intron var., non-coding transcript var.** | 711 | 1506 | 310 | 1993 | 471 | 1771 |
| **Missense var.** | 14 | 42 | 5 | 45 | 8 | 36 |
| **Missense var., NMD transcript var.** | - | 2 | - | 3 | - | 2 |
| **Missense var., splice region var.** | - | 3 | 5 | 5 | - | 7 |
| **Non-coding transcript exon var.** | 41 | 85 | 17 | 113 | 36 | 109 |
| **Regulatory region var.** | - | 3 | - | - | 1 | 2 |
| **Splice region var., intron var.** | 5 | 16 | - | 14 | - | 8 |
| **Splice region var., intron var., NMD transcript var.** | - | 2 | - | 5 | - | 5 |
| **Splice region var., intron var., non-coding transcript var.** | - | 4 | - | 6 | - | 2 |
| **Splice region var., non-coding transcript exon var.** | - | 2 | 3 | 4 | - | 2 |
| **Splice region var., synonymous var.** | - | - | - | - | - | 7 |
| **Stop gained** | 1 | - | 1 | - | - | 4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Stop gained, NMD transcript var.** | - | - | - | - | - | 2 |
| **Stop lost** | - | 1 | - | 1 | - | - |
| **Synonymous var.** | 36 | 57 | 8 | 43 | 19 | 73 |
| **Synonymous var., NMD transcript var.** | 1 | 2 | - | - | - | 2 |

Furthermore, the unique Glycolysis genes were detected in each pool of variants (genes with mutations only in one of the two groups compared) for every comparison (Oligozoospermic vs Normozoospermic, Asthenozoospermic vs Normozoospermic and Teratozoospermic vs Normozoospermic). The results are presented in *Table 17* and *Figure 18*.

*Table 17: Number of unique Glycolysis genes for every comparison performed (Asthenozoospermic vs Normozoospermic, Oligozoospsermic vs Normozoospermic, Teratozoospermic vs Normozoospermic)*

| | **Asthenozoospermic vs Normozoospermic** | | **Oligozoospermic vs Normozoospermic** | | **Teratozoospermic vs Normozoospermic** | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Number of unique Glycolysis genes (total)** | 6 | 4 | 1 | 1 | 0 | 22 |

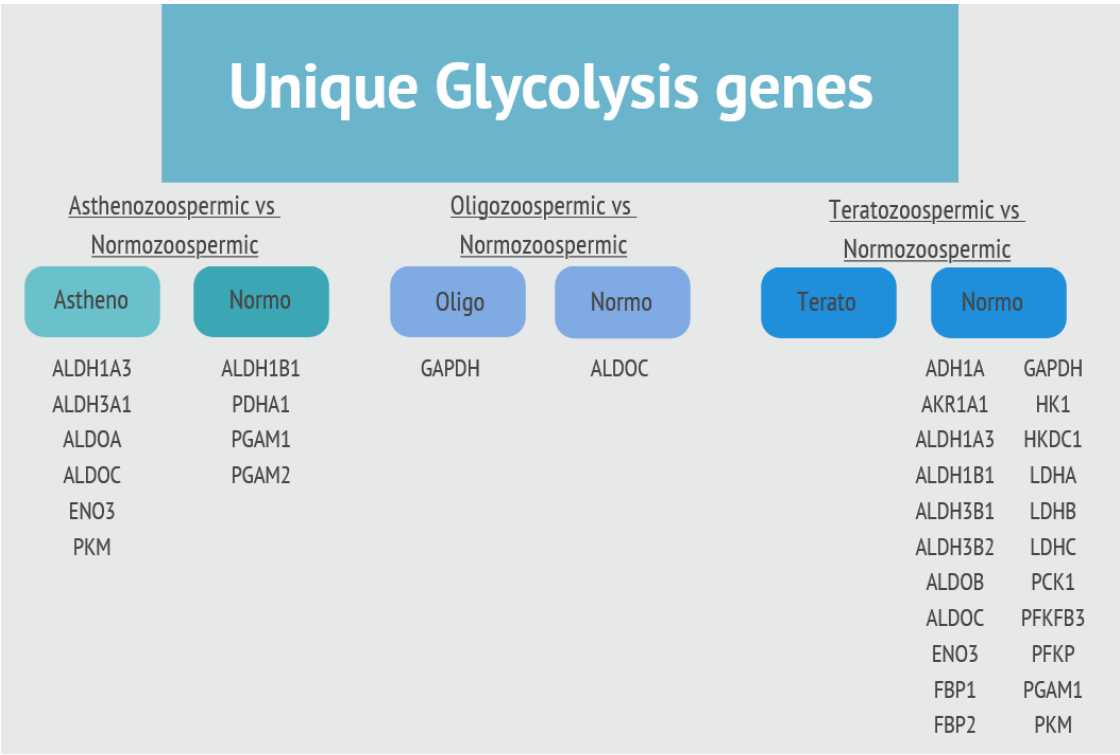


*Figure 18: Unique Glycolysis Genes for every comparison and group studied.*

*OXPHOS Pathway*

In the second part of WGS Analysis, variants found on both nuclear and mitochondrial-encoded OXPHOS genes of samples characterized as normozoospermic or non-normozoospermic were studied.

As it has already been described, at first, variants present only on one group (Cases or Control) for each comparison (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic and Teratozoospermic vs Normozoospermic) were identified (*Table 18*). Moreover, from the total SNPs found on every group, variants located only on OXPHOS genes were detected. Thus, the number of variants found on OXPHOS genes for every group studied and for every comparison is presented also in *Table 18*.

*Table 18: Total variants and variants found on OXPHOS genes for the three comparisons (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic)*

| | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Total SNPs** | 1917404 | 6687079 | 2025316 | 6634959 | 1736484 | 6825542 |
| **OXPHOS SNPs** | 1309 | 8859 | 2011 | 4495 | 1763 | 6721 |
| **OXPHOS/Total SNPs (%)** | 0.07 | 0.13 | 0.1 | 0.07 | 0.1 | 0.1 |

Functional annotation of the unique variants found on OXPHOS genes was also performed using the VEP tool to identify novel and existing variants. The results for every group and every comparison are presented in *Table 19*. Moreover, VEP was also used to study if any variant was associated according to previous research with male infertility or similar phenotype. None of the variants was associated with the phenotype of interest (*Table 19*).

*Table 19: Novel and existing variants found on OXPHOS genes for every group studied and every comparison. None of them was also found associated with the phenotype of interest according to VEP*

| | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Novel variants** | 136 | 460 | 111 | 335 | 25 | 377 |
| **Existing variants** | 1173 | 8399 | 1900 | 4160 | 1738 | 6344 |
| **Phenotype of interest** | - | - | - | - | - | |

The functional annotation of variants found in OXPHOS genes and regarding their consequence is also presented in *Table 20*. As observed, most of the variants are found in intronic regions.

*Table 20: Functional annotation of variants found on OXPHOS genes*

| | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| **Consequences** | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **3 prime UTR variant** | 25 | 56 | 13 | 54 | 13 | 70 |
| **3 prime UTR variant, NMD transcript var.** | 4 | 9 | 1 | 11 | 2 | 7 |
| **5 prime UTR var.** | 4 | 10 | 6 | 12 | 8 | 15 |
| **5 prime UTR variant, NMD transcript var.** | - | 3 | - | 1 | 2 | 1 |
| **Frameshift var.** | 1 | 3 | - | - | - | - |
| **Intron var.** | 696 | 4463 | 1225 | 2346 | 1076 | 3774 |
| **Intron variant, NMD transcript var.** | 253 | 1414 | 331 | 996 | 288 | 1280 |
| **Intron variant, non-coding transcript var.** | 266 | 2635 | 379 | 937 | 336 | 1412 |
| **Missense var.** | 16 | 21 | 14 | 16 | 1 | 20 |
| **Missense variant, NMD transcript var.** | 2 | 1 | 3 | - | - | - |
| **Non-coding transcript exon var.** | 27 | 154 | 26 | 61 | 22 | 71 |
| **Splice region var., intron var., NMD transcript var.** | - | 2 | - | - | - | - |
| **Splice region var., intron var., non-coding transcript var.** | 1 | 3 | - | - | - | - |
| **Stop gained** | - | - | 1 | - | - | - |
| **Stop lost** | - | 1 | - | - | - | - |
| **Synonymous var.** | 14 | 72 | 9 | 54 | 11 | 68 |
| **Synonymous variant, NMD transcript var.** | - | 2 | 2 | 6 | 2 | 2 |

Moreover, as it has already been described, oxidative phosphorylation involves five protein complexes (Complex I-V). Thus, variants can be further categorized according to the mitochondrial complexes. For every comparison, results are presented in *Figures 19-21*.

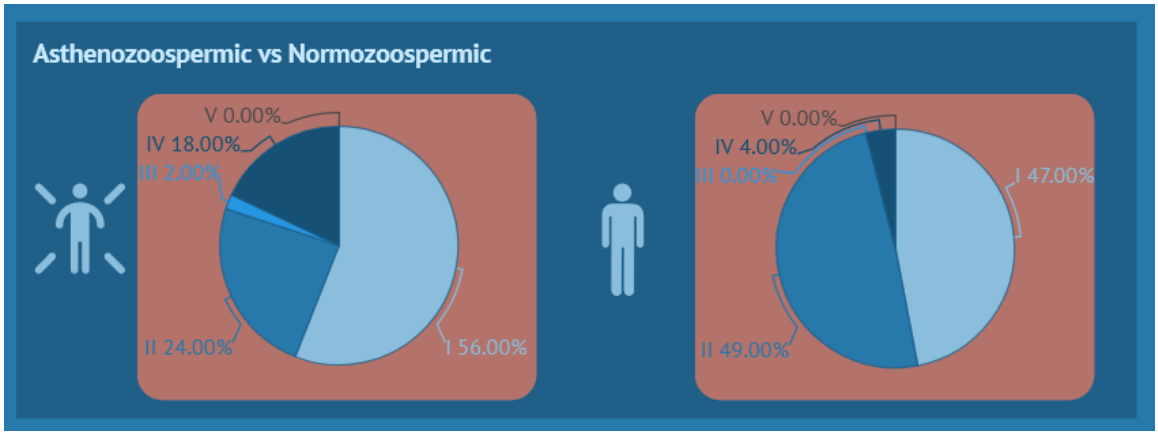


*Figure 19: Categorization of the unique variants according to the mitochondrial complexes (Asthenozoospermic vs Normozoospermic). Results from asthenozoospermic individuals are presented at left, while variants found only on normozoospermic individuals are at right.*

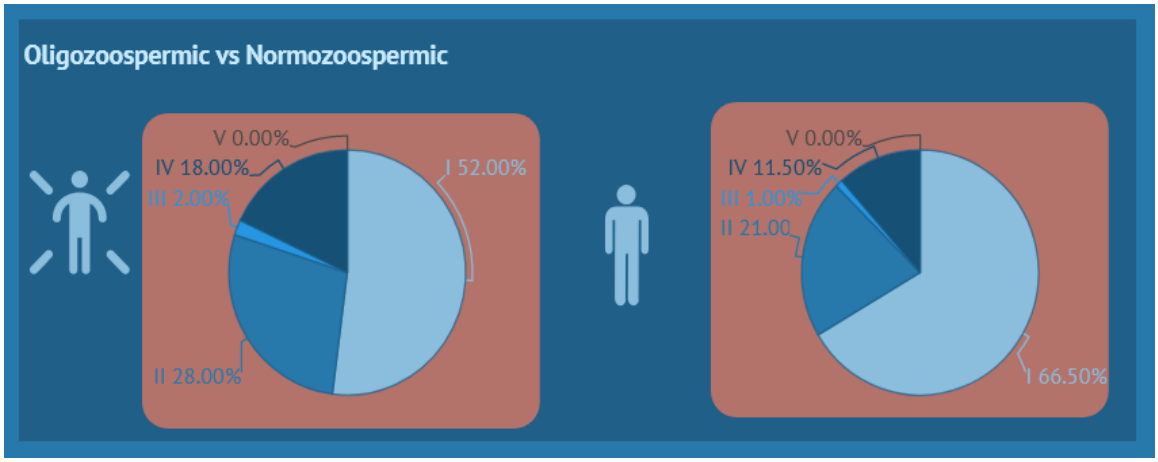


*Figure 20: Categorization of the unique variants according to the mitochondrial complexes (Oligozoospermic vs Normozoospermic). Results from oligozoospermic individuals are presented at left, while variants found only on normozoospermic individuals are at right.*

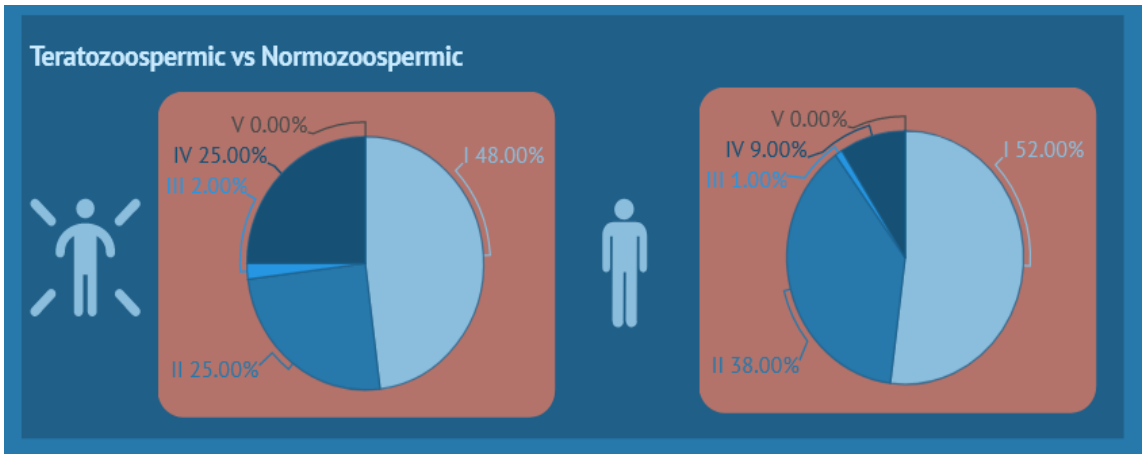


*Figure 21: Categorization of the unique variants according to the mitochondrial complexes (Teratozoospermic vs Normozoospermic). Results from teratozoospermic individuals are presented at left, while variants found only on normozoospermic individuals are at right.*

Furthermore, as has already been described, the proteins that build up mitochondrial complexes are encoded by both mitochondrial and nuclear genes. Thus, variants found on OXPHOS genes could be further characterized as variants found on nuclear or mitochondrial-encoded genes, as presented in *Table 21*, for every comparison.

*Table 21: Variants categorized according to nuclear or mitochondrial-encoded genes and mitochondrial complexes for every comparison and every group*

| | Asthenozoospermic vs Normozoospermic | | Oligozoospermic vs Normozoospermic | | Teratozoospermic vs Normozoospermic | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **OXPHOS variants** | 1309 | 8859 | 2011 | 4495 | 1763 | 6721 |
| **Mt-Complex I** | 12 | 9 | 2 | 14 | 1 | 14 |
| **Nuclear-Complex I** | 723 | 4183 | 1037 | 2975 | 849 | 3473 |
| **Mt-Complex II** | - | - | - | - | - | - |
| **Nuclear-Complex II** | 315 | 4353 | 560 | 944 | 436 | 2586 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Mt-Complex III** | 2 | 3 | 1 | 5 | 0 | 5 |
| **Nuclear-Complex III** | 20 | 8 | 38 | 39 | 30 | 39 |
| **Mt-Complex IV** | 3 | 3 | 1 | 2 | 2 | 3 |
| **Nuclear-Complex IV** | 234 | 298 | 371 | 515 | 445 | 600 |
| **Mt-Complex V** | 0 | 1 | 1 | 1 | 0 | 1 |
| **Nuclear-Complex V** | 0 | 0 | 0 | 0 | 0 | 0 |
| **Total Nuclear variants** | 1292 | 8843 | 2006 | 4473 | 1760 | 6698 |
| **Total mt variants** | 17 | 16 | 5 | 22 | 3 | 23 |
| **mt / OXPHOS variants (%)** | 1.3 | 0.18 | 0.25 | 0.49 | 0.17 | 0.34 |
| **Nuclear/OXPHOS variants (%)** | 98.7 | 99.82 | 99.75 | 99.51 | 99.83 | 99.66 |

Finally, the unique OXPHOS genes were detected in each pool of variants (genes with mutations only in one of the two groups compared) for every comparison (Oligozoospermic vs Normozoospermic, Asthenozoospermic vs Normozoospermic and Teratozoospermic vs Normozoospermic). The results are presented in *Table 22* and *Figure 22*.

*Table 22: Unique OXPHOS genes categorized according to mitochondrial complexes for every comparison performed (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic)*

| | **Asthenozoospermic vs Normozoospermic** | | **Oligozoospermic vs Normozoospermic** | | **Teratozoospermic vs Normozoospermic** | |
|---|---|---|---|---|---|---|
| | **Astheno** | **Normo** | **Oligo** | **Normo** | **Terato** | **Normo** |
| **Complex I unique genes** | 3 | 8 | - | 3 | - | 14 |
| **Complex II unique genes** | - | - | - | - | - | 1 |
| **Complex III unique genes** | 1 | - | - | - | - | - |
| **Complex IV unique genes** | 1 | 3 | - | 2 | - | 4 |
| **Complex V unique genes** | - | 1 | - | - | - | - |
| **Number of unique OXPHOS genes (total)** | 5 | 12 | 0 | 5 | 0 | 19 |

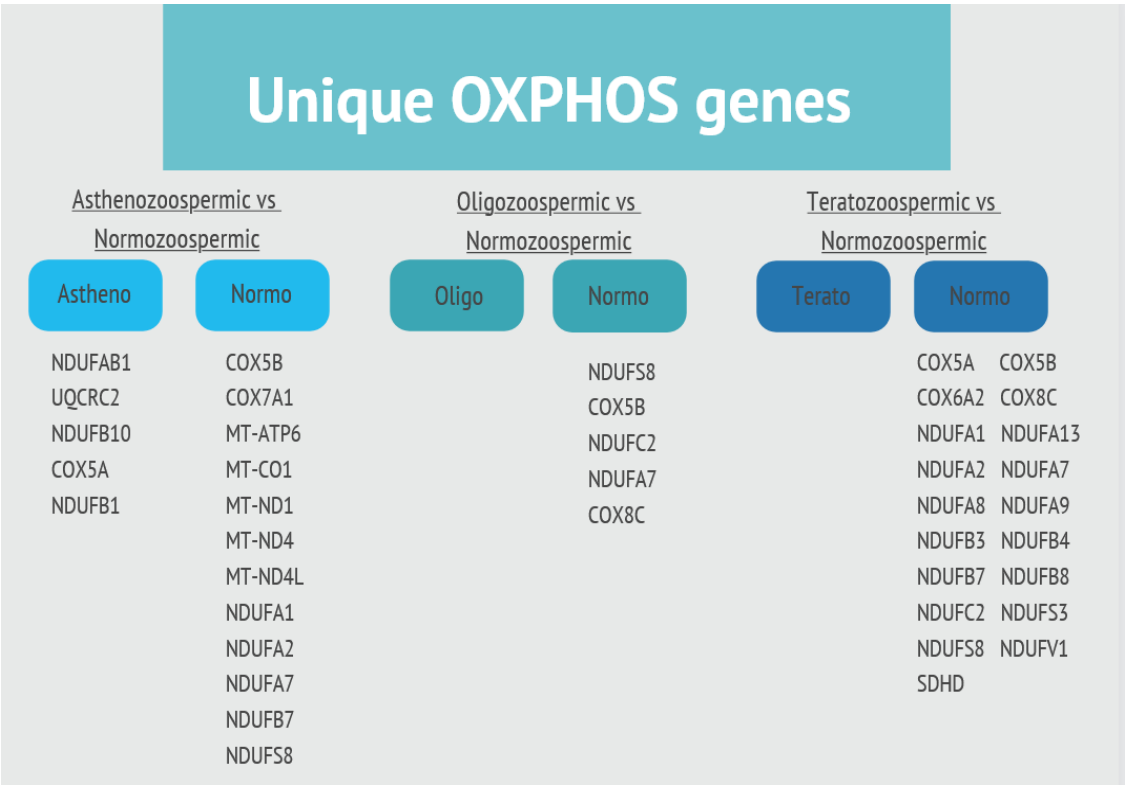


*Figure 2216: Unique OXPHOS Genes for every comparison and group studied.*

# Discussion-Conclusions

Fertilization in mammals is a complex process that requires the synchronization of many cellular events such as motility, capacitation, hyperactivation, and acrosome reaction. Moreover, the site of semen deposition is far from the site of fertilization in the female genital tract (Du Plessis et al., 2015; Visconti, 2012). Thus, energy metabolism is a key factor supporting sperm function as all these actions are energy-dependent and Adenosine Triphosphate (ATP) is required (Du Plessis et al., 2015).

The two main pathways used for ATP production in cells are Glycolysis and Oxidative phosphorylation (OXPHOS) (Du Plessis et al., 2015; Misro & Ramya, 2012; C. Mukai & Travis, 2012). More specifically, glycolysis is an anaerobic process that consists of several stages in order ATP to be produced (Berg et al., 2012) and takes place mainly in the flagellum of spermatozoa (Du Plessis et al., 2015; Visconti, 2012). OXPHOS is also one of the most important pathways in all cells that occurs in mitochondria and is considered to be a more efficient metabolic process for ATP production. OXPHOS takes place in the midpiece of spermatozoa, where mitochondria are found (Du Plessis et al., 2015).

There has been a lot of debate about the role of Glycolysis and OXPHOS in sperm cells as studies show that their dysregulation could affect events essential for the spermatogenesis or the fertilization process, and thus, lead to male infertility (Du Plessis et al., 2015; Misro & Ramya, 2012; Rajender et al., 2010; Visconti, 2012).

In this study, semen and blood samples from normozoospermic and non-normozoospermic men of the Greek population were used for DNA genotyping and GWAS, as well as for Whole Genome Sequencing (WGS), to identify and list variants associated with male infertility in Glycolysis and OXPHOS genes.

More specifically, in Part A, GWAS analysis was conducted by performing four comparisons (Normozoospermic vs Non-Normozoospermic, Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normoozspermic). For every comparison, from all the SNPs that were found as statistically significant, only SNPs found on glycolysis and OXPHOS genes were used for further evaluation.

More specifically, in the first comparison (Normozoospermic vs Non-normozoospermic), the control group consisted of 278 normozoospermic individuals and the case group of 298 non-normozoospermic individuals. After quality control, 488 individuals (control group, n= 248; case group, n= 240) were used for the GWAS analysis. Finally, 17 SNPs were identified as statistically significant on Glycolysis and 12 SNPs on OXPHOS genes. Most of them were found in intronic regions and many of these intronic regions are NMD targets. Nonsense-mediated mRNA decay (NMD) has been described as a mechanism for degradation of aberrant mRNAs that harbor a premature translation termination codon (PTC), but it also targets ‘normal’ mRNAs (with no PTC). NMD may be part of a post-transcriptional mechanism for regulation of gene expression but the molecular mechanism of NMD is only partially understood

(Colombo et al., 2017). One of the SNPs on glycolysis genes (rs1154469) was also characterized as a splice region variant. Some of the SNPs on OXPHOS genes were also characterized as missense variants or found on 5' upstream or 3' downstream regions. Regarding SNPs found on glycolysis genes, many of them were found associated with the acquired immunodeficiency syndrome and SNPs found on OXPHOS genes have been associated with prostate cancer, acquired immunodeficiency syndrome, or aging (telomere length). Previous studies have also associated male infertility with an increased risk for prostate cancer and suggest that male infertility could be a risk marker for later disease (Sharma & Jayasena, 2019; Walsh et al., 2010). Interestingly, the telomere length could be an important biomarker for several diseases, including male infertility, as it has been observed that males with infertility have a shorter sperm telomere length (Amir et al., 2020; Vasilopoulos et al., 2019). Regarding oxidative phosphorylation, most of the SNPs were found on genes encoding for proteins that build up Mitochondrial Complexes I, IV, and V.

In the second comparison (Asthenozoospermia vs Normozoospermia), 270 normozoospermic men were the control group and 5 asthenozoospermic men were the case group. After quality control, 242 individuals (control group, n= 237; case group, n= 5) were used for the GWAS analysis. Finally, 11 SNPs were identified on glycolysis and 12 SNPs on OXPHOS genes. Most of them were found in intronic regions and they are NMD targets, too. A statistically significant SNP on OXPHOS genes was also a missense variant while another was a 5' UTR variant. Most of the SNPs on glycolysis genes were associated with acquired immunodeficiency syndrome while SNPs on OXPHOS genes were, as previously, associated with prostate cancer, acquired immunodeficiency syndrome, or aging (telomere length). Regarding SNPs on OXPHOS genes, these were found on genes encoding for proteins building up all the mitochondrial complexes. This is not a surprise as in general, asthenozoospermia is the most widely studied subcategory of male infertility that has been associated with mutations in OXPHOS genes and especially mtDNA OXPHOS genes (Holyoake et al., 2001; Shamsi et al., 2008).

In the third comparison (Oligozoospermic vs Normozoospermic), 270 normozoospermic (control group) and 57 oligozoospermic (case group) individuals were selected for genotyping, and after that, quality control was performed. After quality control, 327 (control group, n=237; case group, n=52) individuals were used for GWAS analysis. 16 SNPs were identified on glycolysis genes and 10 SNPs on OXPHOS genes. Most of them were intronic but also some NMD and synonymous variants were found as statistically significant. 5' upstream variants were also detected on OXPHOS genes. Regarding association with other diseases, SNPs on glycolysis genes were found associated with acquired immunodeficiency syndrome and age at menarche/menopause in women. Furthermore, SNPs on OXPHOS genes were found to be associated with prostate cancer, acquired immunodeficiency syndrome, or aging (telomere length). As in the first comparison, SNPs of oxidative phosphorylation were found on OXPHOS genes encoding for proteins that build up Mitochondrial Complex I, IV, and V.

In the last comparison (Teratozoospermic vs Normozoospermic), the control group consisted of 270 normozoospermic individuals and the case group of 51 teratozoospermic individuals. After DNA genotyping and quality control, 286 individuals (control group, n=237; case group= 49) were used for GWAS analysis. 21 SNPs were found on glycolysis genes and 7 SNPs were also detected as statistically significant on OXPHOS genes. Regarding SNPs on glycolysis genes, most of them were intronic and NMD variants but a missense and a 3' prime UTR variant were also detected. Moreover, most of the SNPs on OXPHOS genes were intronic. Furthermore, most SNPs on glycolysis genes were associated with acquired immunodeficiency syndrome but one of them was also associated with sudden infant death syndrome (SIDS). SIDS is the unexplained death of a baby less than a year old that might be associated with defects in the infant's brain. A 3' prime UTR variant found on *ALDH1B1* was also associated with varicose veins. Varicose veins are usually observed in legs but when they are found in testicles, they can affect male infertility. The association between varicocele and male infertility is further analyzed in the *Introduction*. Furthermore, the small number of SNPs identified as statistically significant for teratozoospermia on OXPHOS genes were found to be associated with prostate cancer, acquired immunodeficiency syndrome, or aging (telomere length).

Some of the SNPs on glycolysis and OXPHOS genes were found as statistically significant in more than one comparison but none was identified as significant in all of the four comparisons performed. It is also an interesting fact that none of the SNPs found on OXPHOS, or glycolysis genes were previously reported in GWAS studies as associated with male infertility. Moreover, all the genes on which the SNPs were found were searched to detect if a previous association with male infertility has been reported according to other GWAS studies and SNPs on them, but no association was found. Thus, it is the first time that these SNPs are found as associated with male infertility or its subcategories. The same is observed for the genes of glycolysis and OXPHOS in which the SNPs are found.

Finally, an overview of the results regarding the association between male infertility or subcategories of male infertility and SNPs on Glycolysis and OXPHOS genes are presented in *Figure 23 and Figure 24*, respectively.

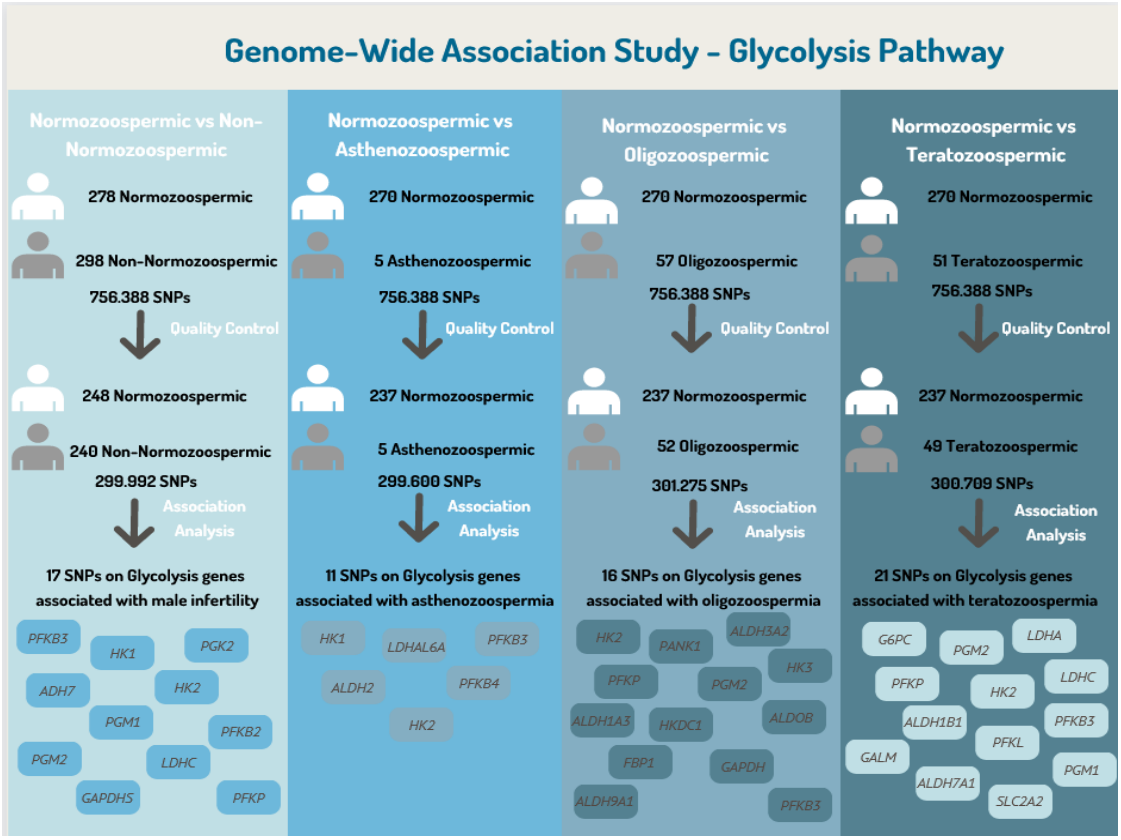


*Figure 23: Overview of the GWAS results for the Glycolysis pathway.*

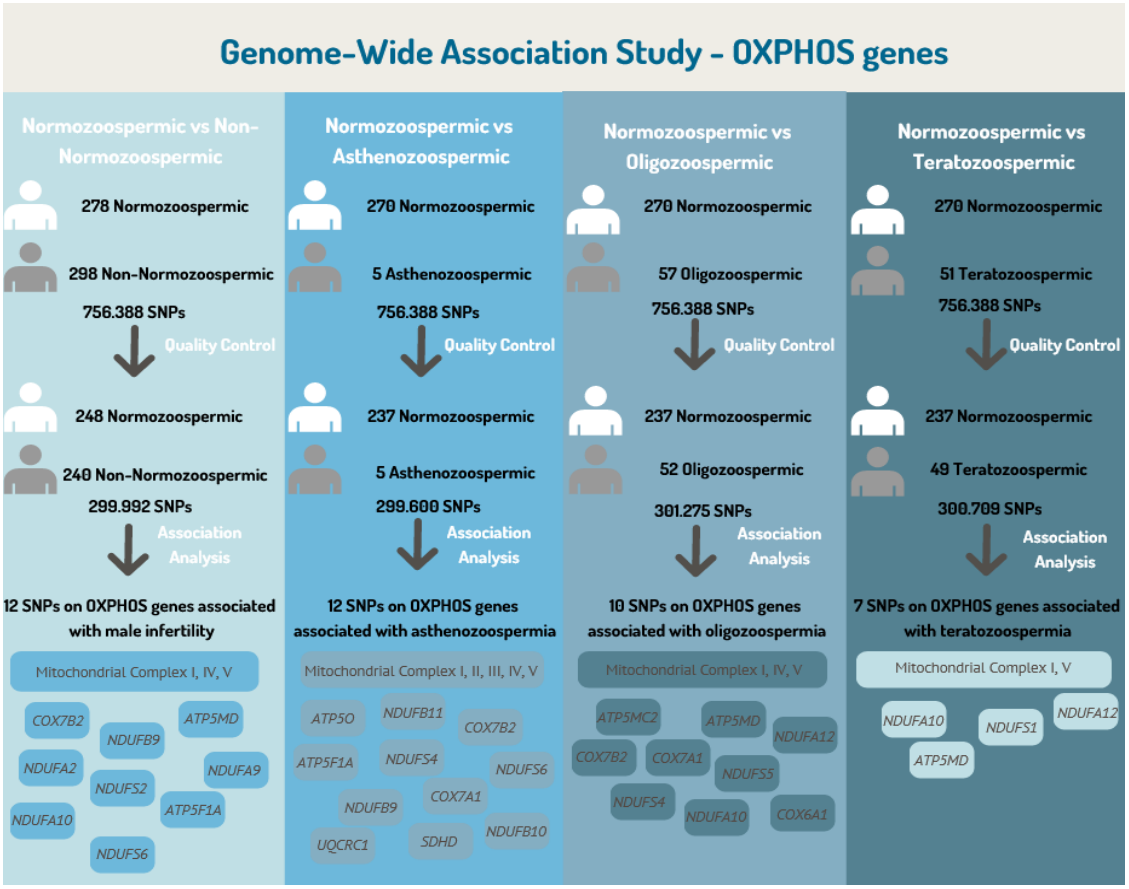


*Figure 24: Overview of the GWAS results for OXPHOS genes.*

Except for GWAS analysis, WGS analysis was also used as genome-wide association studies have some limitations. More specifically, they require large sample sizes, and they typically explain a small proportion of the heritability of the traits and of the observed phenotypic variation. Rare variants are overlooked, too (Singer et al., 2016; Tam et al., 2019). As seen in *Figure 25*, the discoveries that can be made using GWAS analysis can be represented by a big iceberg. Only a small portion of these GWAS studies is revealed, representing GWAS discoveries that have already been made, while the largest part of the iceberg is still uncovered. GWAS studies today are mainly performed on European populations and for easy-to-measure phenotypes. However, GWAS could provide even more results of high quality by their application on more diverse populations and ethnic groups, using larger sample sizes, and different approaches and methods.

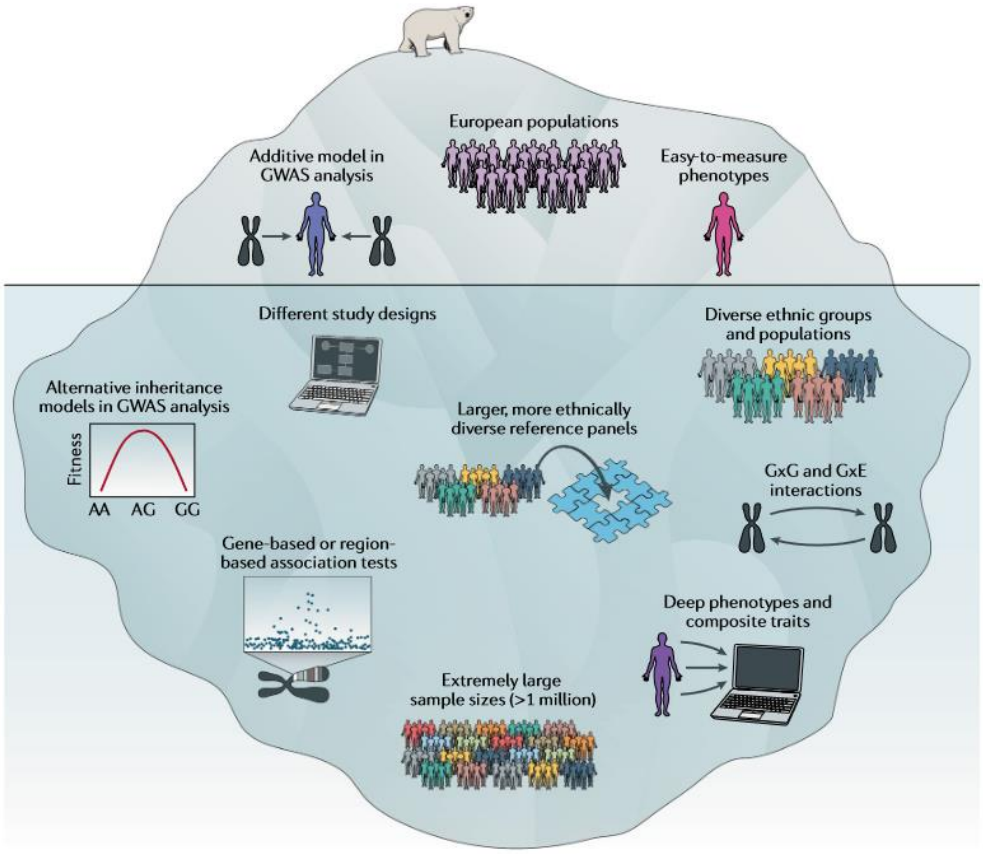


*Figure 25: Discoveries that could be made using GWAS methodology are represented by an iceberg. The part of the iceberg that is above water refers to discoveries that have already been made, but unfortunately, the largest part of it remains still uncovered. GxG, gene–gene; GxE, gene–environment* (Tam et al., 2019).

All these limitations can be transcended by using Whole-Genome Sequencing (WGS). The high cost of WGS is still a limiting factor in many cases but for this reason, pooling sequencing techniques have been developed. These require multiple samples that are pooled together and thus, only a single library per pool is constructed and used for sequencing (Anand et al., 2016). Moreover, it is suggested that even WGS of low depth can reveal more variants than a typical GWAS approach (Gilly et al., 2019).

Thus, in Part B, variants on glycolysis and OXPHOS genes were studied using Whole-Genome Sequencing (WGS) analysis.

At first, variants found on Glycolysis genes were studied by performing three comparisons (Asthenozoospermic vs Normozoospermic, Oligozoospermic vs Normozoospermic, and Teratozoospermic vs Normozoospermic) to identify unique variants found only on one group of every comparison (not shared).

As it was expected, when the percentage of unique Glycolysis variants/Total unique variants was compared for every group, the greatest was found for asthenozoospermic individuals in the comparison of Asthenozoospermic vs Normozoospermic (0.172%). In contrast, very few unique Glycolysis variants were found on both groups of Teratozoospermic vs Normozoospermic comparison (0.001%). According to research, teratozoospermia is associated with changes in the glycolysis pathway as on patients increased glucose levels are observed, but the number of studies linking teratozoospermia and glycolysis pathway is very limited (Mehrparvar et al., 2020). Moreover, it should also be noted that among asthenozoospermic individuals, 196 variants were characterized as novel variants. A great number of novel variants was identified among all the comparisons for non-normozoospermic individuals, too. Regarding their functional annotation, as in GWAS results, most of the variants were found on intronic regions.

Then, unique Glycolysis genes were detected in each pool of variants (genes with mutations only in one of the two groups compared) for every comparison. In the first comparison (Asthenozoospermic vs Normozoospermic), on asthenozoospermic individuals, 6 unique genes were identified. Two of them (*ALDH1A3, ALDH3A1*) belong to the Aldehyde dehydrogenases (ALDH) superfamily which consists of 19 genes. ALDH1A3 plays an important role in glycolysis metabolism via catalytic metabolism of acetaldehyde to acetate (Duan et al., 2016). Moreover, the important biological function of ALDH is proved by several mutations in these genes that lead to their inactivation and the presence of pathological phenotypes. Many of these genes are also associated with cancer and in particular, *ALDH1A3* and *ALDH3A1* are found to be overexpressed in patients with prostate cancer and they seem to play an important role in disease progression (Ibrahim et al., 2018). As it has already been stated, prostate cancer has been associated with male infertility and maybe they both act as risk factors of one for the other (Sharma & Jayasena, 2019; Walsh et al., 2010). Among other unique genes, *ALDOA* and *ALDOC* were identified, as well as *ENO3* and *PKM*. According to research, *ENO3* is a highly expressed gene in spermatogonia while *PKM* exerts differential expression between fertile and infertile men with elevated levels of oxidative stress (Park & Pang, 2021).

Regarding WGS results for OXPHOS genes, the percentage of unique OXPHOS variants/Total unique variants did not exert significant differences between different groups and comparisons as it ranged from 0.07-0.13%. Novel and existing variants were also detected. 136 novel variants were found on the asthenozoospermic group (Asthenozoospermic vs Normozoospermic) and 111 were found on the

oligozoospermic (Oligozoospermic vs Normozoospermic). Only 25 novel variants were found on the teratozoospermic group (Teratozoospermic vs Normozoospermic). As a result of the functional annotation, it is also observed that most of the variants are on intronic regions. Variants were further characterized according to nuclear or mitochondrial-encoded genes. Most of them were found on nuclear-encoded genes but many unique variants found on asthenozoospermic individuals were also located in mt-encoded genes. As it seems, mutations and SNPs in mtDNA have been associated with reduced sperm motility and asthenozoospermia (Abd Elrahman et al., 2021; Kao et al., 2004). Another study suggests also that mtDNA mutations contribute to the etiopathology of male infertility as deletions and mutations in COX-II, ATPase 6 and 8 disrupt ATP production and lead to spermatogenesis arrest and reduced sperm motility (Shamsi et al., 2008). On the other hand, the efficiency of the spermatogenic process measured by sperm cell concentration in the ejaculate (oligozoospermia) has been correlated with the nuclear-encoded mitochondrial enzyme activities (Ruiz-Pesini et al., 2000).

Finally, unique OXPHOS genes were detected in each pool of variants (genes with mutations only in one of the two groups compared) for every comparison. Among groups of non-normozoospermic individuals, unique OXPHOS genes were identified only on asthenozoospermic men. More specifically, five genes were detected. These were *NDUFAB1*, *NDUFB1, NDUFB10, COX5A* and *UQCRC2*. UQCRC2 is a component of ubiquinol-cytochrome c reductase complex and a protein that has attracted researcher's interest regarding male infertility in several cases. It is known that UQCRC2 is associated with sperm dysfunction and spermatogenesis. According to research, nutlin-3a is an antagonist of mouse double minute 2 repressor (MDM2) that activates p53 and induces apoptosis responsible for spermatogenesis. Thus, problems associated with male fertility are observed. Researchers suggest that nutlin-3a causes this decrease in male fertility via UQCRC2 (Shukla et al., 2013). Another study proposes the use of this protein's expression levels in capacitated spermatozoa as a sensitive biomarker for the assessment of male infertility in boar (Kwon et al., 2015). Downregulation of UQCRC2 and mitochondrial dysfunction was also recently validated by a proteomic comparison between normozoospermic and asthenozoospermic testicular cancer patients (Panner Selvam et al., 2019). Moreover, COX5A is a component of the cytochrome c oxidase the last enzyme in the mitochondrial electron transport chain and it seems to be expressed in the head of epididymal sperm but not in testicular sperm (Suryawanshi et al., 2011).

Thus, regarding the strengths and limitations of this study, WGS was used complementary to overcome GWAS limitations that had already been discussed, but it should also be noted that one important parameter is the rigorous quality control applied for the GWAS analysis. More specifically, the exclusion of SNPs and samples that could affect the analysis and lead to false-positive or false-negative results was performed. We should also keep in mind that in some of the comparisons performed the number of samples was very limited (Asthenozoospermic vs Normozoospermic).

In conclusion, a large number of variants was identified on glycolysis and OXPHOS genes by both the approaches used. In particular, in the WGS analysis, the great number of unique variants on Glycolysis genes for the asthenozoospermic group, as well as the identification of unique Glycolysis genes in the same group, some of which have been associated with male infertility in the past, highlights the important role of the glycolysis pathway on sperm motility. The important role of OXPHOS genes in male infertility is also proved by the results of both GWAS and WGS analysis. Moreover, the present study is significant as, for the first time, it explores genetic variants in two of the most important bioenergetics pathways, glycolysis and OXPHOS, associated with male infertility and specific subtypes (oligozoospermia, asthenozoospermia, and teratozoospermia) in the Greek population. Given the growing cases of male infertility worldwide, the study of spermatogenesis is extremely important as there is a growing need to obtain useful applications in clinical practice like diagnostic accuracy and successful treatment.

Regarding future directions, it should be noted that studies recently show that except for glycolysis and OXPHOS, other mechanisms also play an important role in energy production and thus, may have a potential role in male infertility. More specifically, fatty acid β-oxidation (FAO) seems to be involved in energy production during spermatozoa maturation in the epididymis (Asghari et al., 2017). Moreover, researchers used metabolomics approaches to study human sperm cells and found that besides the carbohydrate pathway, lipid and lipoprotein pathways are the most significantly enriched ones (Paiva et al., 2015). Thus, fatty acid metabolism may actively participate in energy metabolism and sperm motility.

Other pathways and mechanisms that contribute to sperm bioenergetics and need further exploration for their role in male infertility are the citric acid cycle and proteins that act as transporters (Asghari et al., 2017). The citric acid cycle or Krebs cycle is a pathway used to generate energy from the oxidation of acetyl-CoA. According to studies, there is an association between the citric acid cycle and asthenozoospermia as gene deficiencies can lead to decreased motility (Martínez-Heredia et al., 2008). The solute carrier family (SLCs) are membrane proteins that transport different metabolites across the mitochondrial membrane and are also required for ATP production in mitochondria. Since they are mitochondrial transporters, it is reasonable to suggest that several mutations causing their inactivity may lead to asthenozoospermia, as their inhibition can reduce the fluxes of several pathways. Recently it was discovered that *Slc22a14* deficiency can result in decreased motility in mice (Kuang et al., 2021). Sugar transporters, sodium-dependent glucose transporters (SGLT), and glucose transporters (GLUT) are membrane proteins that facilitate the transport of glucose across the plasma membrane and they may also contribute to the phenotype of male infertility according to recent studies (Bucci et al., 2011; Pastuszak et al., 2018).

Therefore, it seems that there are many more to reveal behind the mechanism of sperm bioenergetics and male infertility.

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